# Protective Humoral Immune Responses Against Ovine Abortifacient *Chlamydia psittaci*

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## Table of Contents

## List of Tables and Figures

### List of Tables

**List of Figures**

## List of Abbreviations

| | |
|---|---|
| **A** | aborted |
| **AA** | amino acid |
| **ATP** | adenosine triphosphate |
| **C** | cytosine |
| **CMI** | cell mediated immunity |
| **CRP** | cystein-rich protein |
| **CTM** | Chlamydia transport medium |
| **DNA** | deoxyribonucleic acid |
| **DO** | deoxycholate |
| **EB** | elementary body |
| **Fab** | fragment antigen binding |
| **Fc** | fragment crystalline |
| **fMOMP** | "Fusion" MOMP |
| **FPLC** | Fast protein liquid chromatography |
| **G** | Guanine |
| **GAG** | Glycosaminoglycan |
| **GPIC** | guinea pig inclusion conjunctivitis |
| **GST** | Glutathione transferase |
| **H-2** | Major histocompatability complex (murine) |
| **HI** | Heat inactivated |
| **HRP** | Horseradish peroxidase |
| **Hsp** | heat shock protein |
| **i.p** | Intraperitoneal |
| **i.v.** | Intravenous |
| **IFN-γ** | Interferon gamma |
| **ifu** | Inclusion forming units |
| **Ig** | Immunoglobulin |
| **IL** | Interleukin |
| **L** | Lambed |
| **LGV** | lymphogranuloma venereum |
| **LPS** | lipopolysaccharide |
| **M∅** | macrophage |
| **M/A** | Marcol/Arlacel A |
| **MAB** | monoclonal antibody |
| **MHC** | major histocompatability complex |
| **MIF** | microimmunofluorescence |
| **mMOMP** | "membrane" MOMP |
| **NS** | non-significant |
| **OEA** | ovine enzootic abortion |
| **OMP** | outer membrane protein |
| **PAGE** | polyacrylamide gel |
| **PBL** | peripheral blood leukocytes |
| **PBS** | phosphate buffered saline |
| **PID** | pelvic inflammatry disease |
| **PL-F** | phagosome-lysome fusion |
| **PMNL** | polymorphonuclear leukocyte |
| **R-LPS** | rough LPS |
| **RB** | reticulate body |
| **RNA** | ribonucleic acid |
| **S-LPS** | smooth LPS |
| **sem** | standard error of the mean |
| **SNA** | serum neutralisation assay |
| **Tc** | T-cytotoxic cell |
| **Th** | T-helper cell |
| **tMOMP** | "truncated" MOMP |
| **TNF** | tumour necrosis factor |
| **VS** | variable segment |

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## Declaration

The work presented in this thesis was part of a larger project concerned with the investigation of recombinant antigens of the major outer membrane protein of *Chlamydia psittaci,* with respect to protection of sheep against chlamydial abortion. A full role was undertaken in the design of the experiments described and in the interpretation of results. Where applicable, input from colleagues has been fully acknowledged.

# CHAPTER 1

## LITERATURE REVIEW

## Literature Review

### Historical context

The genus *Chlamydia* is the only genus in the family *Chlamydiaceae* and the order, *Chlamydiales*. Much confusion has surrounded the classification of *Chlamydiae*, which were originally classed as protozoa and then later, after their isolation in the early 1930's (Bedson *et al.*) as viruses due to their small size. Unlike viruses, however, *Chlamydiae* possess both types of nucleic acids, RNA and DNA, and multiply by binary fission rather than self-assembly.

*Chlamydiae* are distinguished from other organisms by several criteria, most significantly by their unique developmental lifecycle and the fact that due to their inability to synthesise high-energy compounds such as ATP, they can only survive by intracellular parasitism of host cells. In addition, *Chlamydiae* have been found to have several genus specific antigens, including the lipopolysaccharide moiety of the outer membrane which shows similarities to the outer membranes of Gram-negative bacteria. However, unlike Gram-negative bacteria, *Chlamydiae* do not contain detectable peptidoglycan.

The genus was originally separated into two species, namely *Chlamydia trachomatis* and *Chlamydia psittaci*, as determined by morphological and chemical characteristics in cell culture and by host specificities. Whilst *C. trachomatis* can be identified by iodine positive staining of compact cell inclusions indicating the presence of glycogen and by its sensitivity to sulphadiazine antibiotics, *C. psittaci* is sulphadiazine resistant and produces diffuse cell inclusions which did not stain with iodine. Species host specificities are also obvious. Whereas *C. trachomatis* was exclusively a human pathogen, with the exception of one strain which infects mice under experimental conditions (Mouse pneumonitis), *C. psittaci* strains infect only animals (Table 1).

Until recently, all non-human isolates have been classed as *C. psittaci* strains giving the species high variation in DNA homology, unlike *C. trachomatis* which shows

considerable DNA relatedness within the species. Over the last seven years, however, two new species have been accepted, both of which have arisen from isolates originally classed as *C. psittaci*. The first new species, *C. pneumoniae*, is now considered as a genuine human respiratory pathogen and not a zoonosis. Its proposal was based on the unique ultrastructure of the infectious particle, as well as DNA analysis and serology. *C. pneumoniae* showed 94% or greater DNA relatedness within the species and only 10% or less with the other two species of *Chlamydia*. Later, a second new species was proposed, *C. pecorum*, to incorporate all non-abortion, non-conjunctival mammalian strains. Although it was morphologically similar to *C. psittaci*, *C. pecorum,* like *C. pneumoniae*, could be distinguished by DNA analysis and immunological assays.

**Table 1.1.** Characteristics of the four species of Chlamydia (Fukushi and Hirai, 1993).

| | Species | | | |
|---|---|---|---|---|
| Characteristics | *C. pecorum* | *C. psittaci* | *C. pneumoniae* | *C.trachomatis* Trachoma/LGV |
| Natural hosts | Cattle,sheep | Birds, lower mammals | Humans | Humans |
| Morphology of EB | Round | Round | Pear-shaped | Round |
| Morphology of inclusion | Oval,dense | Variable,dense | Oval,dense | Oval,vacuolar |
| Glycogen in inclusion | No | No | No | Yes |
| Folate biosynthesis | No | No | No | Yes |
| N°. of serovars | 3 | ? | 1 | 12/3 |
| Mol. % G+C of DNA | 39.3 | 39.6 | 40.3 | 39.8 |
| DNA homology % to | | | | |
| *C. pecorum* | 88-100 | 1-20 | 10 | 1-10 |
| *C. psittaci* | | 14-95 | 1-8 | 1-33 |
| *C. pneumoniae* | | | 94-96 | 1-7 |
| *C. trachomatis* | | | | 92 |

Development of new powerful detection techniques focusing on DNA sequence comparisons and the use of monoclonal antibodies in microimmunofluorescence (MIF) have allowed more accurate typing of chlamydial isolates. In fact it has been suggested that further species divisions within *C. psittaci* may be appropriate with the continued application of gene analyses and the use of monoclonal antibodies to identify common antigens. At present, *C. trachomatis* is known to have 15 serovars, A, B, Ba, C, D, E, F, G, H, I, J, K, L1, L2, L3 which are divided between three distinct biovars; the oculogenital biovar, the lymphogranuloma venereum (LGV) biovar and the mouse pneumonitis biovar (MoPn). Serovars A to K belong to the oculogenital biovar, whilst L1, L2 and L3 are members of the LGV biovar. Mouse pneumonitis contains a single strain and therefore makes up only one biovar. However recently, a new nonhuman chlamydial strain, SFPD, was isolated from hamsters (Stills et al, 1991), and found to be closely related to *C. trachomatis*. This new strain may belong to a rodent group which would also comprise the mouse biovar. *C. psittaci* comprises 4 biovars; GPIC, the agent of guinea pig conjunctivitis; avian strains; feline pneumonitis and abortifacient strains which infect a diverse range of mammals.

**Diseases caused by Chlamydia**

*C. trachomatis,* the larger species of the two human pathogens, infects primarily the ocular and genital mucosal epithelium. Serovars A, B, Ba and C infect the eye, whilst serovars D to K are responsible for infection of the genital mucosae. Although *C. trachomatis* often produces asymptomatic infections, acute infections may occur resulting in conjunctivitis, urethritis or pelvic inflammatory disease (PID). Chronic infections are exemplified by chronic salpingitis, which results in infertility or ectopic pregnancy due to blockage of the fallopian tubes. Progression of *C. trachomatis* in the eye to a chronic inflammatory state, leads to the development of trachoma. This is the major cause of preventable blindness in the world, with most cases concentrated around developing countries in parts of Asia, Africa and South America. Sexually transmitted *C. trachomatis* is responsible for epidemic disease in

the United States of America and in Europe, causing an estimated 4.5 million infections annually (Wyrick *et al*, 1993; Hook *et al*, 1994). *C. trachomatis* also causes a systemic disease, lymphogranuloma venereum (LGV) by infection of the inguinal or pelvic lymph nodes (Grayston and Wang, 1975). This is a far more invasive sexually transmitted disease infecting both lymphoid and epithelial cells (Schachter, 1990) and leading to considerable necrosis and formation of abscesses. Infection by biovar LGV organisms stimulates granuloma formation in which mononuclear phagocytes undergo transformation into epithelioid cells.

*C. pneumoniae* contains only one strain, previously known as TWAR. It is a human pathogen causing a 'flu-like illness with a long-lasting dry cough. It has been shown to be responsible for both endemic and epidemic pneumonia (Grayston *et al.* ,1986; Kleemola *et al.*, 1988; Saikku *et al.*, 1985). At the peak of an epidemic it can account for 45% of adult pneumonia cases (Leinonen *et al.*, 1990). Typically, 5-7% of adult respiratory infections can be attributed to *C. pneumoniae* (Marrie *et al.*, 1987; Grayston *et al.*, 1990). Recently, the presence of antibodies against *C. pneumoniae* has been associated with the occurrence of coronary heart disease (Kuo *et al.*, 1993; Saikku *et al.*, 1988; Thom *et al.*, 1992), sarcoidosis ( Gronhagen-Riska *et al.*, 1988) and asthma (Hahn *et al.*, 1991).

*C. pecorum* is an animal pathogen recently differentiated from *C. psittaci* strains by DNA analyses and serology ( Fukushi and Hirai, 1992). It is known at present to infect cattle, sheep and swine ( Fukushi and Hirai, 1992 and Kaltenboeck *et al.*, 1993), causing pneumonia, polyarthritis, encephalomyelitis and diarrhoea.

*C. psittaci* can be divided into mammalian and avian groups. The avian strains cause a zoonotic human disease called psittacosis (from the Latin 'psittacine' - parrot) (Harris, 1983 ; Grimes, 1987; Macfarlane and Macrae, 1983), which is manifested as a mild 'flu-like infection or as a more serious atypical pneumonia requiring patient hospitalisation (Shewan, 1980). Transmission of infection occurs by inhalation of dried faecal dust which is contaminated with the organism. Mammalian hosts form a

diverse group, including sheep, cattle, goats, cats, dogs, guinea pigs, muskrats and koalas (Girjes *et al.*, 1993). Mammalian isolates of *C. psittaci* cause guinea pig inclusion conjunctivitis (GPIC) in experimentally infected animals (Rank and Sanders, 1992), feline pneumonitis and ruminant abortions, in particular ovine enzootic abortion (OEA). Not only is OEA a major cause of economic loss to the sheep industry (Linklater, 1979), but it also has important ramifications as a zoonotic disease causing systemic disease and abortion in pregnant women (Beer *et al.*, 1982). Confirmation of the association between *C. psittaci* and spontaneous abortion in women was first documented in 1985 (Johnson *et al.*, 1985) when a farmer's wife who had been in contact with infected sheep during the 20th to 28th week of pregnancy aborted in the 29th week following 4 days of fever, nausea, vomiting and severe headaches. The causative agent, *C. psittaci* was isolated from the placenta and foetus.

Abortifacient *C. psittaci* infection in sheep is characterised by a Chlamydia-induced placentitis which results either in abortion in late pregnancy or in premature lambing (Stamp *et al.*, 1950; Storz, 1988). Infection and abortion can occur in the same pregnancy (Blewett *et al.*, 1982), or more usually naive animals are exposed to chlamydiae-infected tissues in one season and subsequently abort in the next lambing season. The intermittent period between infection and abortion still remains obscure, with chlamydiae detectable only by methods which do not rely on cultivation of chlamydiae *in vitro* (Papp *et al.*, 1993). It appears that chlamydiae are mobilised from their site of latency at some as yet undetermined stage of pregnancy (Wilsmore *et al.*, 1984; Huang *et al.*, 1990) and are detectable in the placenta by culture at approximately 60 days of gestation (dg). Infection becomes established primarily in the placentome from where it gradually spreads forming visible lesions in the intercotyledonary areas (Buxton *et al.*, 1990; Papp *et al.*, 1993). Although placental infection occurs earlier, pathological changes are not apparent until the third trimester of pregnancy (Buxton *et al.*, 1990) and it is probably as a result of these changes to the maternal-foetal junction that foetal death occurs. The extent of pathological changes in the foetus varies between different species, with only minor effects

apparent in lambs compared to more serious pathological changes in bovine foetal tissue.

As a result of chlamydial abortion, dams develop considerable immunity to placental infection and are generally resistant to further chlamydial abortions. It is possible, however, that low levels of *C. psittaci* persist in immune animals and some evidence suggests that chlamydiae may be excreted from the reproductive tract during subsequent oestrus cycles (Papp *et al.,* 1994), allowing transmission of chlamydiae to other ewes during breeding. Whilst rams do become infected with *C. psittaci,* causing epididymitis, periorchitis and poor quality semen (Storz *et al.,* 1976; Rodolakis and Bernard, 1977), there is no real evidence that sexual transmission contributes to spread of infection to a significant degree (Linklater, 1996).

## Lifecycle of *Chlamydia*

*Chlamydiae* have a unique biphasic lifecycle of which part must be completed within eukaryotic cells. The cycle is best described by dividing it into several stages according to the process(es) occurring at that time, namely: attachment to the host cell; subsequent uptake; inhibition of phagolysosomal fusion; reorganisation of the particle; multiplication and finally expulsion from the host cell. These stages will be discussed individually following a brief overview.

Two distinct morphological structures exist representing the two major phases of the lifecycle. These are the elementary body (EB) and the reticulate body (RB). Many intermediate stages are passed through in the progression from one form to the other. The EB is an extracellular, infectious form, which is metabolically inactive and contains a highly condensed chromosome. Rigid disulphide linking of the outer membrane holds the EB at a diameter of around 300 nm. This measurement increases to approximately 800-1000 nm as the EB reorganises its outer membrane structure, relaxing the condensed chromatin to form the metabolically active,

vegetative RB. Division of the RB proceeds by binary fission and after several rounds, some of the RBs begin a transformation back to the EB form. These events occur within a host cell-membrane-bound vacuole and as more intermediate and infectious forms appear, the size of the vacuole increases, pushing the host cell cytoplasm to one side until eventually the vacuole bursts, releasing its contents into the extracellular space. The mature EBs are free to infect further host cells and the cycle continues.

**i) Attachment**

The attachment mechanisms by which chlamydiae gain entry into the host cell are unknown. Since both chlamydial cell and host cell surfaces have acidic net charge, receptor-mediated interaction between surfaces is needed for the attachment of *Chlamydia*. It is likely that surface structures of the organism are involved in adhesion to the host cell membrane and most likely are of proteinaceous nature, given the loss in infectivity after heat-treatment of chlamydiae (Peeling and Brunham, 1991). Notably heat-treatment affects attachment of trachoma biovar D and not the LGV biovar, L2, indicating that different modes of attachment may be available to chlamydiae. It has been speculated that the function of the major outer membrane protein (MOMP), is that of an adhesin. Other functions of the MOMP have since been investigated and these will be discussed later.

Viable chlamydiae are not required for attachment to host cells, since EB envelopes can elicit not only attachment, but also endocytosis and inhibition of lysosomal fusion (Eissenberg, 1983). Pre-formed components on the chlamydial outer membrane are therefore presumably responsible for the major events in infection. Treatment of host cells with trypsin and other proteases results in almost complete loss of attachment of EBs, for as long as regeneration of receptors is inhibited (Byrne *et al.*, 1976). This information, together with that of Byrne and Moulder (1978), Hatch *et al.* (1981) and Bose and Paul (1982) describing loss of EB attachment by gentle heat treatment of the EBs, suggests that the chlamydial adhesin is most likely a

protein. Evidence supporting a role for MOMP in the attachment process (Su *et al.*, 1988 and 1990; Caldwell and Perry, 1982; Lucero and Kuo, 1985), is strengthened by the finding that cleavage of variable segment 2 (VS2) by trypsin-treatment results in reduced attachment of EBs to HeLa cells (Su *et al.*, 1988). Again, the inhibition is serovar-specific, with loss of attachment of *C. trachomatis* serovar B but not L2.

More recently, Zhang and Stephens (1992) proposed a novel mechanism of chlamydial attachment in which a glycosaminoglycan (GAG) bridge is formed between receptors on both the chlamydial and host-cell surfaces. It is proposed that chlamydiae are able to synthesise a molecular mimic of heparan sulphate and use this to attach to host-cells via heparan sulphate receptors as seen with the pseudorabies virus (Mettenleiter *et al.*, 1990). This GAG-dependent mechanism of infection has been proposed for both trachoma and LGV biovars (Zhang and Stephens, 1992; Chen and Stephens, 1994), although only the former shows inhibition of attachment as well as infectivity by a specific inhibitor, heparan sulphate. Eukaryotic cells have been shown to endocytose free heparan sulphate by a receptor-mediated mechanism (Barzu *et al.*, 1985). Utilisation of such a mechanism which does not normally result in phagolysosomal-fusion might explain the lack of fusion when the same receptor is used by chlamydiae. The structure of the Chlamydia-synthesised GAG is similar to that of other GAGs i.e. a linear polysaccharide chain consisting of disaccharide repeats (Jackson *et al.*, 1991). This is consistent with the sensitivity of chlamydial attachment to periodate-treatment.

Furthermore, it was recently shown that the MOMP of chlamydiae is glycosylated (Swanson and Kuo, 1991) and the glycan-moiety is involved in the attachment process of *C. trachomatis* to HeLa cells (Swanson and Kuo, 1994). A second glycoprotein of 32KD has also been implicated in chlamydial attachment (Swanson and Kuo, 1994). Other proteins with this molecular mass include adhesins (Hackstadt *et al.*, 1996; Wenman and Meurer, 1986) and histone-like proteins (Hackstadt *et al.*, 1993), although the latter have a higher isoelectric focusing point

(pI) compared to the 32KD glycoprotein. Other glycoproteins present in a range of bacteria have been shown to act as adhesins (Sharon, 1984; Olden *et al.*, 1985).

The process of chlamydial attachment therefore appears to be multifactorial and probably depends not only on the chlamydial strain, but also on the specific host-cell type.

**ii) Entry**

Similarly to attachment of chlamydiae to host-cells, uptake of chlamydiae is likely to occur by more than one mechanism. Phagocytosis has been shown to occur predominantly when chlamydiae are centrifuged onto host-cells (Reynolds and Pearce,. 1991), a method of infection widely used in various laboratories. Chlamydial (*C. trachomatis*, L1) ingestion can be impaired by the microfilament inhibitor cytochalasin D and microtubule disrupters, vincristin and vinblastin, but not by inhibitors of receptor mediated endocytosis (monodansyl cadaverine, amantadine) (Ward and Murray, 1984) indicating a role for phagocytosis with centrifugation. However, under static conditions, receptor-mediated endocytosis appears to be the preferred mechanism of internalisation. The relevance of each of these processes to the natural mode of infection is unknown, however it has been speculated that direct cell-to cell transfer may be possible due to the force of expulsion of EBs from infected cells.

Hodinka *et al.* (1988) showed that chlamydiae entry into host cells involves microvilli, coated pits, coated vesicles and endosomes which are also characteristics of receptor-mediated endocytosis entry mechanisms. Transmission electron microscopy revealed that chlamydiae preferentially bind to pre-existing cell-surface microvilli and travel down these structures to their base, where they enter the host cell through specialised invaginated pits of the plasma membrane. Chlamydia appear to be associated, however, with both uncoated and coated (clathrin) pits (Ward and Murray, 1984; Hodinka and Wyrick, 1986; Wyrick *et al.*, 1989). Upon

internalisation, EBs are surrounded by membrane-bound vesicles which do not fuse with host cell lysosomes (Friis, 1972; Eissenberg and Wyrick, 1981; Eissenberg *et al*, 1983).

**iii) Inhibition of phagolysosomal fusion**

Once internalised, chlamydiae reside within host-cell membrane-bound vacuoles. These endosomes are able to avoid acidification and do not fuse with lysosomes allowing the chlamydiae to complete their developmental cycle in a protected intracellular environment. The mechanism by which chlamydiae are able to inhibit phagolysosomal-fusion (PL-F) is unknown. However, Eissenberg *et al.* (1983) demonstrated that EB envelopes were capable of inhibiting PL-F and intact chlamydiae were not essential. Initially, binding to host cell microvilli may reduce electrostatic repulsion between electro-negative membranes of the chlamydiae and host, allowing more specific receptor-mediated attachment to occur. Eukaryotic cells naturally utilise receptor-mediated endocytosis to ingest nutrients and regulatory components (Goldstein *et al*, 1979; Silverstein *et al*, 1977) and only some of these are destined to fuse with lysosomes. Thus, it is possible that chlamydiae utilise an inherent endocytic mechanism which does not result in phagolysosomal-fusion.

Recently, Schramm *et al.* (1996) proposed that chlamydiae-filled endosomes were able to avoid PL-F by retaining membrane characteristics of early endosomes. It has been shown that only mature endosomes are able to fuse with lysosomes (Mullock *et al.*, 1989, Mullock *et al.*, 1994), thus chlamydiae would be protected for as long as the early endosome was prevented from developing further. Chlamydial components are incorporated into both the inclusion (endosome) and host-cell membranes during chlamydial development (Hackstadt *et al.*, 1994) and it is possible that these may be involved in maintaining the early characteristics of endosomal membranes. There is evidence that a protein (Inc A) specifically synthesised by RBs is transported to the inclusion membrane (Rockey *et al.*, 1995). However, whilst Inc A is probably involved in some aspect of inclusion membrane establishment, maintenance or

functional modification, further work is required to determine its purpose unequivocally.

**iv) Metabolism**

Chlamydiae are known as "energy parasites" requiring host-cell association to survive and develop. Thus, the biosynthetic capacity of chlamydiae is much reduced compared to bacteria in general. The loss of biosynthetic capacity may be an explanation for the unusually small chlamydial genome, $1x10^6$ bp which is only a quarter of the size of the *Escherichia coli* genome. Chlamydiae are therefore restricted to an intracellular habitat and rely on the host cell for many of its nutrients (amino acids, nucleotides, nucleotide-sugars and fatty acids) to construct macromolecules (protein, DNA, RNA, lipids, LPS and murein) as well as to provide ATP as an energy source. It has been demonstrated, however, that whilst chlamydiae have evolved mechanisms to reduce energy-costs to themselves by obtaining metabolic products from the medium, it appears that under certain circumstances of nutrient deprivation, chlamydiae regain the ability to manufacture for example folates in species which normally transport the thymidine nucleotide precursor from exogenous sources. Such circumstances render the species involved, *C. psittaci* (except strain 6BC) or *C. pneumoniae* unusually sensitive to sulphonamides, an inhibitor of *de novo* folate biosythesis.

Transfer of cell-derived nutrients must essentially occur through three membranes; the inclusion membrane, and the chlamydial inner (cytoplasmic) and outer membranes. An alternative hypothesis suggests that chlamydial surface projections, which appear to pass through the inclusion membrane, may allow direct transfer of factors from the host-cell cytoplasm into the chlamydial inclusion. In addition to this arrangement, the porin-like activity of MOMP may behave as a non-specific nutrient channel, allowing passive diffusion from the nutrient rich host cytoplasm to the hypotonic environment of the chlamydial periplasm. Movement of components across the chlamydial cytoplasmic membrane may involve specific carrier-mediated

transport systems, which would explain the existence of a proton-motive force found by Hatch *et al.* (1984).

Chlamydiae must, however, retain the ability to manufacture macromolecules unique to bacteria and therefore are capable of limited protein, RNA and DNA synthesis once supplied with the basic components and ATP. This has been demonstrated by the inhibition of eukaryotic metabolism but not chlamydial growth, by cycloheximide or emitine which are specific eukaryotic inhibitors. Genes encoding enzymes which are involved in LPS synthesis have been identified. Other compounds not found in host-cells, but present in chlamydiae are branched-chain fatty acids and phosphatidylglcerol, necessary for fatty acid and lipid syntheses, respectively. Thus, pathways involved in the syntheses of these compounds must also be retained by chlamydiae. Species differences are again apparent in metabolic processes, *C. trachomatis* can synthesise glycogen, whist *C. psittaci* can not. Tryptophan requirements also appear to depend on individual strains.

**v) Release**

Todd and Caldwell (1985) described a mechanism of infectious EB release from host cells by exocytosis, enabling the host cell to remain intact. In contrast, rupture of degenerate infected cells showing vesiculation of the endoplasmic reticulum and loss of ribosomes and microvilli (Todd *et al*, 1976) was suggested to be a more natural scenario by Benes (1986) who observed explosive release of EBs from infected cells. This was suggested as the mechanism of cell-to-cell transmission *in vivo,* in strains which do not require centrifugation to assist infection *in vitro*.

## Structure of the Cell Envelope

As discussed above, survival of chlamydiae depend upon successful attachment and entry into host cells. The structure of the cell envelope is therefore of great

importance, since it is this region of the organism which is involved in host cell to bacterial cell contact. The following paragraphs describe the structure of the cell envelope in some detail..

The envelopes of both EBs and RBs share some characteristics with those of Gram-negative bacteria. Both possess an outer and an inner cytoplasmic membrane, each of which contributes 3 layers to the overall cell envelope. At this point, however, chlamydiae diverge from the typical Gram-negative morphology and no evidence of a peptidoglycan layer has been found ( Caldwell *et al.,* 1981; Manire and Tamura, 1967), nor has anyone been able to detect any N-acetyl muramic acid (Matsumoto, 1988; Barbour *et al.,* 1982; Garrett *et al.,* 1974) both of which are commonly found in Gram-negative bacterial envelopes. Penicillin-binding proteins are, however, present and render the chlamydiae susceptible to penicillin treatment (Barbour *et al.,* 1982).

Surface projections have been identified which associate with DNA fibrils radiating from the nucleus and extend approximately 20 nm above the outer membrane (Matsumoto, 1982). Each EB has approximately 22 projections, clustered in a polarised fashion. Similarly, RBs have been shown to have varying numbers of projections, ranging from approximately 45 at 10 hours after infection, to 20 at later stages (20 hours) of development.

In addition, it is proposed that a lipopolysaccharide (LPS) moiety, sensitive to periodate treatment (Barwell, 1952) nestles in the outer membrane and is exposed towards the outer surface (Birkelund *et al.,* 1988). The LPS is composed of lipid A and a saccharide portion (Reitchel and Brade, 1992) which contains a trisaccharide of 3-deoxy-D-manno-octulosonic acid (Kdo) of the sequence αKdo(2→8)-αKdo(2→4)- αKdo (Brade *et al.,* 1987; Holst *et al.,* 1993) and is therefore of the rough type (R-LPS). Recently, an alternative form of LPS was isolated from chlamydiae grown in yolk sacs of embryonated eggs (Lukacova *et al.,* 1994). This LPS was shown to share properties with those of LPS from enterobacterial wild-type

strains (smooth form- S-LPS). Both types of LPS have a common 2,8-linkage, unique to chlamydial LPS, whereas the 2,4-linkage is responsible for serological cross-reactions between chlamydiae and other Gram-negative bacteria.

**Proteins of the cell envelope**

Chlamydial proteins contained in the cell envelope can be separated into two fractions by sarkosyl (detergent) treatment (Filip *et al.*, 1973; Caldwell *et al.*, 1981). Many proteins are soluble in sarkosyl, notably the 62 KD and 45 KD proteins. The sarkosyl insoluble fraction consists primarily of proteins of 12-15, 40 (MOMP) and 60 KD (Hatch *et al.*, 1984; Newhall, 1987; Sardinia *et al.*, 1988). Both the 12-15 (OMP 3) and 60 (OMP 2) KD proteins are cysteine-rich lipoproteins (CRP) ( Allen *et al.*, 1990; Everett and Hatch, 1991; Everett *et al.*, 1994) which are synthesised late in the developmental cycle (Hatch *et al.*, 1986; Kaul *et al.*, 1990; Newhall, 1987; Sardinia *et al.*, 1988) and therefore are mainly found in EBs rather than RBs (Allen *et al.*, 1990; Batteiger *et al.*, 1985). In contrast, the 40 KD protein which comprises 60% of the total outer membrane protein and is thus termed, the Major Outer Membrane Protein (MOMP) is synthesised predominantly at midcycle, when RBs are dividing. Extensive disulphide-bond cross-linking in EBs between MOMP, OMP 2 and OMP 3 provides both structural rigidity and osmotic resistance to the EBs (Hatch *et al.*, 1984; Hatch *et al.*, 1986; Hackstadt *et al.*, 1985) in much the same way as typical Gram-negative bacterial peptidoglycan. RBs in comparison, are relatively fragile structures. Different forms of MOMP have been found in RBs and EBs with only monomeric MOMP existing in the metabolically active RB, whilst dimeric, trimeric and oligomeric forms have been found in EBs (Newhall and Jones, 1983; McCafferty *et al.*, 1995; de Sa *et al.*, 1995).

Other proteins of interest include the family of heat shock proteins (hsp60 and hsp70) and several adhesins (31 KD and 18KD) also found on the outer membrane (table below). The heat shock proteins are widely conserved amongst chlamydiae and in particular the 57 KD and 75 KD proteins belonging to hsp60 and hsp70, respectively

may be responsible for the hypersensitivity reaction associated with severe sequelae of chlamydial infections (Brunham *et al.*, 1985; Brunham *et al.*, 1986; Brunham *et al.*, 1987; Cerrone *et al.*, 1991). The gene encoding the 57 KD hsp has been shown to have 48% homology to a human hsp (Morrison *et al.*, 1990; Cerrone *et al.*, 1991) and therefore the immune response leading to the formation of scar tissue may potentially be considered as an autoimmune reaction.

**Major serologically defined antigens:** (Brunham and Peeling, 1994).

| Size (KD) | Name | Function/characteristic |
|---|---|---|
| 75 | Dna K | heat shock protein |
| 60 | Gro EL | heat shock protein |
| 57 | OMP 2 | membrane protein |
| 40 | MOMP | membrane protein |
| 32 | HctB | histone |
| 29 | Mip | peptidyl cis/trans isomerase |
| 10 | LPS | Kdo transferase |

**MOMP as an antigen**

In addition to being the major proteinaceous component of the chlamydial outer membrane, MOMP i) is post-translationally glycosylated (Swanson and Kuo, 1994); ii) plays an electrostatic role in adherence (Su *et al.*, 1990); iii) may function as a porin (Bavoil *et al.*, 1984); iv) contains surface-exposed, trypsin-sensitive antigenic epitopes (Baehr *et al.*, 1988); and v) is a major candidate for chlamydial vaccine development (Ward, 1992; Murdin *et al.*, 1993).

MOMP contains a range of epitopes (Caldwell and Schachter, 1982; Zhang *et al.*, 1987) including species- (common to all serovars), sub-species- (common to several serovars) and serotype- (unique to only one serovar) specific epitopes. The serovar and subspecies MOMP determinants are immunodominant (Caldwell *et al.*, 1987) and are highly accessible on the surface of chlamydiae in their native form (Zhang *et*

*al.,* 1987). The exposed epitopes of MOMP have been mapped and shown to be contained in three out of four variable domains (Baehr *et al.,* 1988). In addition, monoclonal antibodies against these surface exposed regions show neutralising ability *in vivo* against chlamydial infection (Caldwell *et al.,* 1987). Baehr *et al.* (1987), proposed a scheme for the arrangement of MOMP in the outer membrane and identified primary areas of species, sub-species and serovar-specific epitopes (Table 1.2).

**Table 1.2:** *Epitope specificities on MOMP (Baehr* et al., *1987)*

| Specificity | Epitope |
|---|---|
| serovar | VS1/VS2 |
| subspecies/species | VS4 |

**The immune response to chlamydial infection**

Although different species display different host cell tropisms, the immune responses triggered by chlamydial infections are notably similar. A general overview will be described followed by a more specific discussion on cell-mediated and humoral factors involved in controlling infection.

An initial acute phase reaction occurs upon invasion of the host by chlamydiae and this is marked by the recruitment of polymorphonuclear leucocytes, mainly neutrophils, to the site of infection. Subsequent sub-acute and chronic stages of infection involve a switch to predominantly lymphocytes with additionally an influx of plasma cells, eosinophils and macrophages (Monnickendam *et al.*, 1980; Kuo, 1988; Buxton *et al.*, 1990; Yang *et al.*, 1994).

Animal models have been used to simulate chlamydial infections in order that the immune response may be studied in more depth. Such models have shown the roles of individual T cell subsets, cytokines and the involvement of humoural immunity in

the host's response to infection. It is generally recognised that infection with chlamydiae results in an initial Th1 response (Kincy-Cain and Rank, 1995), which is characterised by the secretion of interleukins 1, 2 and 8 (IL-1, IL-2, IL-8) and interferon-gamma (IFN-γ). IFN-γ appears to play a pivotal role in bacterial clearance in both *C. trachomatis* and *C. psittaci* (Williams *et al.*, 1989; Zhong *et al.*, 1989; McCafferty *et al.*, 1994) where it has a chlamydiostatic effect on infected cells. In addition, Lyt-$2^+$ (cytotoxic) T cells, produced in response to *C. psittaci* (Lammert, 1982) infection have been shown to be protective in mice (Buzoni-Gatel *et al.*, 1992). Evidence exists, however, for the activation of both cytotoxic and helper T cells in the primary response to chlamydial infection (Stagg *et al.*, 1993).

Despite the fact that CMI has been shown to be critical for the clearance of chlamydial infection (Rank *et al.*, 1985) and may even be capable of resolving infection in the absence of antibody, passive transfer experiments have demonstrated that antibodies against chlamydiae are also able to reduce chlamydial infection (Buzoni-Gatel *et al.*, 1990). In particular, antibodies mapping to linear peptides on the surface exposed variable regions, VS1, 2 and 4, of *C. trachomatis* have been shown to neutralise chlamydial infectivity *in vitro* (Zhong *et al.*, 1989; Peterson *et al.*, 1991; Qu *et al.*, 1993). Surface exposed regions of *C. pneumoniae* in comparison, appear to be located on variable segments 1, 3 and 4 and may rely more heavily on conformational structure for neutralising epitopes (Peterson *et al.*, 1991). In the latter case, contrasting evidence has been documented for the immunodominance of MOMP. Whilst Campbell *et al.* (1990) found only weak seroconversion in humans to the MOMP of *C. pneumoniae,* Peterson *et al.* (1996) were able to select for either high or low MOMP-specific antibody titres in mice, depending on the H-2 genotype of the mouse strain. The importance of the VSs in chlamydial pathogenesis is unclear, although it is logical to assume that due to their surface location and the diversity of VS sequences amongst chlamydial species, that these are areas of probable antigenic shift and as such are of major importance to chlamydial species survival.

**i) Cell-Mediated Immunity**

Cytokines have been extensively studied to elucidate their effects on chlamydial infections. In particular, IFN-γ appears to be a major factor in controlling chlamydial development through its chlamydiostatic effects both *in vitro* (Rothermal *et al.*, 1983; Shemer and Sarov, 1985; Zhong *et al.*, 1989; Rank *et al.*, 1992) and *in vivo* (Williams *et al.*, 1988; Summersgill *et al.*, 1995). IFN-γ is an important lymphokine produced by activated T-cells, which is able to induce microbicidal activity in bacterially infected cells as well as increasing natural killer (NK) cell activity in order to destroy infected cells (Nathan *et al.*, 1983; Byrne *et al.*, 1988; Dijmans *et al.*, 1989; Dijmans *et al.*, 1990). Monoclonal antibodies against IFN-γ cause exacerbation of infection with *C. psittaci* in mice (McCafferty *et al.*, 1994). It is believed that one mode of action of IFN-γ is to deplete tryptophan concentrations in the cell (Taylor and Feng, 1991; Byrne *et al.*, 1986; Carlin *et al.*, 1987; Pfefferkorn and Guyre, 1984; Rapoza *et al.*, 1991). However, chlamydial strains differ with respect to their tryptophan requirements (Allen and Pearce, 1983) and so this may not be a universal mechanism of chlamydial restriction.

Other mechanisms modulated by IFNs include the generation of reactive oxygen intermediates (Nathan *et al.*, 1983), the production of nitrogen intermediates by nitric oxide synthase (Green *et al.*, 1990; Adams *et al.*, 1990), as well as the decyclisation of tryptophan by indoleamine 2,3-dioxygenase (IDO) (Byrne *et al.*, 1986; Pfefferkorn and Guyre, 1984). Furthermore, IDO activity has also been detected after chlamydial infection of human macrophage cultures and found to induce production of type I interferons (IFNα and IFNβ) (Paguirigon *et al.*, 1994), as well as modulate interleukin 1 (IL-1) production (Arenzana-Seisdedos *et al.*, 1985; Gerrard *et al.*, 1987). IL-1 induction by chlamydiae in monocyte-derived macrophages was also shown to occur by Paguirigon *et al.* (1994), Rothermal *et al.* (1989) and Magee *et al.* (1992).

IL-1 is produced not only by macrophages, but also under some circumstances by B-cells, T-cells and fibroblasts (Akira *et al.*, 1990) and may be involved in the activation of T-cells and the induction of cytotoxic T-cell activity (Houssiau *et al.*, 1988 and 1989; Kunimoto *et al.*, 1989; Mizutani *et al.*, 1989). Carlin and Weller (1995) demonstrated that IL-1 was able to enhance IFN-mediated inhibition of chlamydial growth by increasing the amount of IDO activity induced by IFNs. Furthermore, they identified IL-1β as the major contributor, with the activity of IL-1α only minor in comparison. IL-1 may also contribute to the inflammation and fibrosis associated with trachoma and chlamydial tubal infertility (Rothermal *et al.*, 1989; Magee *et al.*, 1992) because of its effects on the production of collagen (Spielvogel *et al.*, 1978) and collagenase (Dayer *et al.*, 1986), as well as on stimulation of fibroblast proliferation (Schmidt *et al.*, 1982).

IL-6 appears to act synergistically with IL-1 in chlamydial infections (Magee *et al.*, 1992) and may be induced not only by IL-1, but also by TNF-α (either by itself or with IFN-γ), by IL-3, platelet-derived growth factor and GMCSF (Akira *et al.*, 1990; Van Snick, 1990). Importantly, it has been proposed that TNF-α , a potent mediator of inflammation, is able to inhibit replication of *C. trachomatis* (Manor and Sarov, 1988; Shemer-Avni *et al.*, 1988 and 1989) and of *C. pneumoniae* (Summersgill *et al.*, 1995). TNF-α production has been demonstrated in response to chlamydial infection both *in vitro* (Williams *et al.*, 1989) and *in vivo* (Williams *et al.*, 1990), although the mechanism of TNF-α production in the latter study was T-cell independent, whilst host resistance against chlamydiae shows a major T-cell dependency (Williams *et al.*, 1981, 1982 and 1984). IFN-γ may therefore be the more important cytokine in controlling chlamydial infection (Byrne *et al.*, 1987 and 1988; Williams *et al.*, 1988).

**ii) Humoral Immunity**

Humoral immune responses are directed against the major surface exposed regions of chlamydiae. Thus, since MOMP is considered to be the major immunodominant

antigen, sera from *C. trachomatis*- and *C. psittaci*-infected individuals not unexpectedly contain antibodies predominantly against epitopes on this protein (Brunham *et al.*, 1987). In addition, considerable seroconversion has been identified against Omp 2, LPS and the two cytoplasmic heat shock proteins, DnaK and GroEL (Brunham *et al.*, 1987). In particular, sera from trachoma patients reacts strongly with VS1 of MOMP and less so against VS2 (Jones, *et al.* 1992). However, since VS2 is the most sequence-variant region of MOMP (Yuan *et al.*, 1990), this would imply that immunoassays may not be able to detect the full complement of VS2 epitopes which in turn indicates a high prevalence of conformational or discontinuous epitopes on VS2. It should be noted however, that the MOMP of *C. pneumoniae* does not appear to be immunodominant (Puolakkainen *et al.*, 1993; Yamamoto *et al.*, 1992) unlike those of *C. trachomatis* and *C. psittaci*. In fact, the species-specificity of *C. pneumoniae* appears to reside on a 35 KD peptide within the 76 KD protein. Antibodies prepared against this protein neutralise the infectivity of *C. pneumoniae* in cell culture (Perez-Melgosa *et al.*, 1994). In general, it is believed that antibodies recognising serovar and subspecies- specific epitopes are protective, whilst those directed against species-specific epitopes are not (Lucero and Kuo, 1985; Zhang *et al.*, 1987).

Numerous *in vitro* analyses have identified species and serovar-specific MOMP-specific monoclonal antibodies (MAbs) which are able to neutralise chlamydial infectivity for cell monolayers (Caldwell and Perry, 1982; Caldwell and Schachter, 1982; Baehr *et al.*, 1988; Lucero and Kuo, 1985; Zhang *et al.*, 1987; Peterson *et al.*, 1988; Zhang *et al.*, 1989; Toye *et al.*, 1990; Peterson *et al.*, 1991; Cheng *et al.*, 1992; Morrison *et al.*, 1992; Su *et al.*, 1990; Qu *et al.*, 1993; Batteiger *et al.*, 1996). However, many variables can be used in performing *in vitro* serum neutralisation assays (SNA) including the cell line, the use of centrifugation, the diluent and the addition of complement (Peeling and Brunham, 1991; Peterson *et al.*, 1988; Su *et al.*, 1991) all of which may affect the outcome of the assay. Indeed such divergent results were reported by different laboratories, that it was necessary to set up a workshop to establish a universal protocol for SNA using *C. trachomatis* (Byrne

*et al.,* 1993). Furthermore, the similarity between *in vitro* and *in vivo* conditions is unknown, so that a protective MAb *in vitro* may be non-protective *in vivo*. The antibody isotype is also important in such cases, since neutralisation by IgG2b or IgG3 antibodies, which are able to bind FcγRIII receptors, may be abrogated by using cells which possess these specific receptors, such as macrophages (Ravetch and Kinet, 1991).

Humoral immunity appears to be important in clearance of chlamydial infection, since antibody produced by experimentally infected animals or naturally infected humans can neutralise both *C. trachomatis* and *C. psittaci* infection *in vitro* and *in vivo* (Howard, 1975; Megran *et al.,* 1988; Nichols, 1973; Reeve and Graham, 1962; Zhang *et al.,* 1987; Buzoni-Gatel *et al.,* 1990). Neutralisation was shown to depend on bivalent IgG binding which caused either loss of EB attachment to host cells possibly by reducing electrostatic attraction or by stearic hindrance of receptor-binding areas, or inhibition of the porin function of MOMP after antibody-coated chlamydiae were internalised (Caldwell and Perry, 1982; Peeling *et al.,* 1984; Su *et al.,* 1990). None of these possibilities have yet been proved. That aggregation of IgG molecules was not a cause of chlamydial neutralisation was shown by Su and Caldwell (1991), who demonstrated that monovalent Fab fragments from two neutralising MAbs were able to prevent chlamydial attachment to host cells.

**Animal models of chlamydial infection**

Animal models are not only useful tools for assessing the virulence of chlamydial strains, but have also allowed greater examination of the immune response to chlamydial infections. The high prevalence of both *C. trachomatis*-related ocular disease and genital infections initiated the development of laboratory models to study these important pathogens. In the first case, primates were used in conjunction with human serovars of *C. trachomatis* (Fraser *et al.,* 1975; Taylor *et al.,* 1982) and the resultant disease was found to be remarkably similar to human trachoma. The cost of primate models is, however, limiting and consequently smaller laboratory animals

were utilised. In such a model, the agent of guinea pig inclusion conjunctivitis (GPIC), a *C. psittaci* strain, was used to simulate human inclusion conjunctivitis in guinea pigs (Murray, 1964; Monnickendam *et al.,* 1980). Not only was acute conjunctivitis produced, but upon repeated infection, a trachoma-like disease was illicited.

As with ocular infections, models based on primates (Patton *et al.,* 1987; Moller *et al.,* 1980; Patton *et al.,* 1983; Wolner-Hanssen *et al.,* 1986; Jonson *et al.,* 1980), cats (Kana *et al.,* 1985), Koalas (McColl *et al.,* 1984), mice (Barron *et al.,* 1981; Tuffrey and Taylor-Robinson, 1981; Tuffrey *et al.,* 1986; Ito *et al.,* 1990) and guinea pigs (Mount *et al.,* 1973; Rank *et al.,* 1979; Rank and Barron, 1983; Batteiger *et al.,* 1993) have been successfully used to study chlamydial genital infections. Although the GPIC strain is a member of *C. psittaci*, it produces infection in the genital tract of guinea pigs which is remarkably similar to human *C. trachomatis* genital infection with respect to pathogenesis, pathology, immunity and ability to be transmitted sexually (Patton and Rank, 1992; Rank and Sanders, 1992). A major advantage of the guinea pig model is that GPIC is a natural pathogen of guinea pigs and so the responses studied can be assumed to be the result of natural host-parasite interactions. In addition, the reproductive system of female guinea pigs is more similar to that of human females both histologically and physiologically, than other rodents. An alternative model has also been developed to study chlamydial genital infections and in this case, mice are infected with *C. trachomatis*, MoPn, the agent of mouse pneumonitis (Barron *et al.,* 1981 and 1984; Pal *et al.,* 1993 and 1994). MoPn is much more virulent in mice than human strains of *C. trachomatis* (Kuo and Chen, 1980), hence the preference for the MoPn strain in this particular model. *C. trachomatis* has been associated with pneumonitis in infants (Beem and Saxon, 1977; Tack *et al.,* 1980). Consequently, a mouse model has been developed which again uses the MoPn biovar of *C. trachomatis* (Kuo and Chen, 1980).

Whilst *C. trachomatis* infections have been extensively studied, interest in *C. psittaci* has only escalated in the last 20 years after an increase in reported cases of ruminant

abortion. Buzoni-Gatel and Rodolakis (1983) developed a mouse model in order to explore the virulences of a number of isolates of *C. psittaci*. They defined virulence as being the capacity of chlamydiae to cross mucosal barriers, to spread beyond lymph nodes, to multiply in the reticuloendothelial system and to reach and colonise target organs. Inoculation of non-pregnant mice via the footpad did not result in the systemic spread of non-virulent (intestinal) strains, whilst virulent (abortifacient) strains were able to progress beyond the lymph nodes and colonise the spleen. Not only did this study highlight an attractive method for distinguishing abortifacient from intestinal strains of *C. psittaci,* but it also demonstrated that infection by abortifacient chlamydiae could be assessed by splenic colonisation.

In the same set of experiments, infection of pregnant mice with abortifacient Chlamydia was also achieved, resulting in death of infant mice *in utero*. It was also observed that intestinal strains were able to induce abortion, but only when extremely large numbers were injected intraperitoneally into mice. Since *C. psittaci* strains have been shown to preferentially inhabit macrophages (Wyrick and Brownridge, 1978), it was suggested that intraperitoneal injection allowed the chlamydiae the opportunity to colonise peritoneal macrophages and thus be hidden from host-immune surveillance and subsequently be disseminated to various organs by a haematogenous route.

The mouse model was further used to characterise the immune response to *C. psittaci* with respect to cell-mediated and humoral immunity (Buzoni-Gatel *et al.,* 1987). Both spleen and liver samples were analysed for viable chlamydiae by plaque assay (Banks *et al.,* 1970) and gave similar results to each other. Control mice were able to completely resolve infection in some instances by day 8 after inoculation and so samples were restricted to the period before this from day 4 to day 6 after inoculation. McEwan and Foggie (1954) originally determined chlamydial infection in mice by monitoring histopathological changes. Visual assessment of lesions, particularly in the lung tissue were performed laboriously under the light microscope.

Whilst Buzoni-Gatel *et al.*, (1987) demonstrated an apparent involvement of both cellular and humoral immunity in the defence against *C. psittaci* infection by passive transfer of both spleen cells and immune mouse sera, McEwan and Foggie (1954) had previously employed passive transfer of sheep serum to determine the immune status of sheep after immunisation with various yolk sac vaccines. In the former case, immune cells were able to lower infection levels in mouse organs by 3-5 log plaque forming units (pfu), whereas immune sera only reduced infection by 1.5 log pfu. Furthermore, transfer of immune cells led to rapid and total eradication of infection, compared to a slower but significant reduction in chlamydiae titre with transfer of immune sera. Cytotoxic T-cells have also been directly shown to control *C. psittaci* infection, both *in vitro* (Lammert, 1982) and *in vivo* (Buzoni-Gatel *et al.*, 1992). In addition, transferred murine polyclonal and monoclonal antibodies were able to lower levels of splenic colonisation by *C. psittaci* (Buzoni-Gatel *et al.*, 1990), however, characterisation of the antibodies in the latter study was not performed and their specificities were unknown.

Mouse models have also been useful in investigating genetic factors which may influence the susceptibilities of individuals to chlamydial infection (Byrne *et al.*, 1990; Fuentes *et al.*, 1990; Tuffrey *et al.*, 1992). Susceptibility to *C. psittaci* infection was shown to depend both on the H-2 haplotype and on the genetic background of the mouse strain (Buzoni-Gatel *et al.*, 1994), whilst the antibody response to the heat shock proteins hsp60 and hsp70 were shown to be H-2 restricted in human chlamydial genital infection (Zhang, 1992). The availability of inbred strains of mice is therefore a major advantage of mouse models of chlamydial disease.

## Vaccination against infection

The first commercial chlamydial vaccine was developed by McEwan and Foggie (1952) in response to the identification of *C. psittaci* as the causative agent of ovine enzootic abortion (OEA). Formalinised, yolk-sac grown material or infected ovine

foetal membranes were originally used. However, this "watery" vaccine (Littlejohn, Foggie and McEwan, 1952) was soon replaced with a more resilient adjuvanted vaccine which abrogated the need to revaccinate ewes on a yearly basis (McEwan and Foggie, 1954). The disease remained under control for approximately 20 years by extensive use of the adjuvanted vaccine.

During the late 1970's however, vaccine efficacy began to breakdown (Linklater and Dyson, 1979) and immunisation with adjuvanted vaccine or primary infection with reference strain H574 was shown to have no protective effect against experimentally infection of ewes with strain S26/3, isolated from an aborted ewe in a vaccinated flock. Subsequent efforts to produce an efficacious vaccine have included the development of an avirulent, live vaccine containing a temperature-sensitive mutant strain of *C. psittaci* prepared by treatment with nitrosoguanidine (Rodolakis and Bernard, 1984). The possibility of reversion of the mutant to a virulent form however, is a major concern with this marketed vaccine.

Jones *et al.* (1995) recently reported good levels of protection in ewes using a semi-purified, tissue-culture grown chlamydial vaccine. In this report, protection was dependent on the vaccine dose administered and similar to the findings of McEwan and Foggie (1954), on the nature of the adjuvant used. Limitations to the production of such a vaccine commercially however, reside in the practical and economic production of large quantities of antigen by tissue-culture.

It had been demonstrated that an outer membrane preparation of chlamydial EBs was able to protect pregnant ewes against OEA (Tan *et al.*, 1990). The main constituent of the preparation was MOMP indicating an important immunoprotective role for this particular protein in OEA. MOMP was therefore investigated for its ability to prevent abortions when presented to ewes as a recombinant vaccine (Jones *et al.*, 1992). Initial studies involving expression of the MOMP sequence as a fusion protein in *E.coli* (termed fMOMP) proved encouraging (Herring *et al.*, 1992; Jones *et al.*, 1992). Construct pD5 contained all 367 amino acids of native MOMP plus 11

additional amino acids derived from the vector and attached to the $NH_2$ terminus. A second construct, pC2 comprised only the last 228 amino acids of the $C_1$ terminus plus 8 residues at the $NH_2$ end. Both recombinants were expressed as inclusion bodies. Repetition of the study using freshly expressed constructs failed to produce any protection against chlamydial infection in ewes. Analysis of the humoral responses against each construct showed a marked lack of antibodies specific to VS2 and VS4 in the second experiment, although overall seroconversion was high. In contrast, seroconversion to abortion resulted in antibodies which reacted with all 4 variable segments by immunoblotting (Herring *et al.*, 1992).

Whilst expression of recombinant proteins by bacteria generally results in a denatured conformation with the formation of insoluble inclusion bodies (Francis, in: Vaccines for Veterinary Applications; Dascher *et al.*, 1993), various chemical treatments can be used to help the protein regain its native conformation. Alternatively, the recombinant protein can be expressed so that it is automatically translocated to the outer membrane of the vector, although problems of toxicity to the vector may arise by accumulation of signal-peptides associated with transport of the recombinant protein. Export of recombinant MOMP to the outer membrane of *E.coli* was achieved and the product designated mMOMP (Herring *et al.*, in preparation). The conformational integrity of the recombinant protein was unknown however. A further adaptation of this construct was made by expression in a PET vector and the product named MOMP19. The most recently produced recombinant construct of MOMP in this project, has been named tMOMP (truncated MOMP) and although expressed as an insoluble inclusion body like fMOMP, it was hoped that this particular construct may be more natively refolded. At the initiation of the present study, only fMOMP had been examined in pregnant ewes.

## Summary and Aims

Vaccination of sheep with inoculum grown in embryonated hen's eggs provided effective protection against ovine enzootic abortion (OEA) for many years. The reason for its loss in efficacy remains unclear, although changes in the dominant field strain of *C. psittaci* may have been partially responsible. The high cost of inoculum production and low economic returns, resulted in withdrawal of the OEA vaccine from the veterinary products market. Subsequent analysis of reported sheep abortions during the last twenty years, indicated an increasing prevalence of *C. psittaci* in upland flocks and generated a new search for an efficacious vaccine.

The criteria for the modern OEA vaccine had altered from those associated with the original egg grown vaccine. Production of large amounts of antigen were required and at relatively low cost to the pharmaceutical industry. Neither egg grown nor tissue culture produced antigen would be able to fulfil these requirements and so alternative approaches to vaccine designs were considered. Amongst the various proposals, an attractive idea was the presentation of chlamydial antigen as a recombinant protein. Other vaccines had been produced as recombinant proteins by expression in various bacterial systems, such as *Escherichia coli*, with great success. Since it would be unnecessary to express a complete bacterial genome as a recombinant protein and unlikely that the bacterial vector could survive such a feat, only those parts of the immunising bacterium which are protective would be selected for expression as a recombinant protein.

In the case of *C. psittaci,* it has been shown that the major outer membrane protein (MOMP) is able to confer immunity on sheep and prevent abortion after experimental exposure to the organism. Furthermore, studies on *C. trachomatis* have indicated that protection may be provided by several epitopes located primarily in regions of MOMP where variable amino acid sequences which give rise to different serovars are found (variable segments 1-4). In *C. psittaci*, no such serovar discrimination exists and whilst sequence variations occur in the variable segments,

these are limited to species differences with *C. trachomatis, C. pneumoniae* and the recently described *C. pecorum.* Thus a single MOMP sequence should be able to provide universal protection against all abortifacient strains of *C. psittaci.* The basis of the present study was therefore:

**Hypothesis:-** Humoral immunity plays a significant role in defence against abortifacient *C. psittaci* infection and is directed against variable segment epitopes located on the major outer membrane protein.

**Aim:-** To identify a protective recombinant protein which may be used as a universal OEA vaccine and to define more clearly the relationship between immunity and the humoral immune response of sheep to *C. psittaci* infection.

**Approach:- 1)** To develop an *in vitro,* or an animal model which could illustrate protection against abortifacient *C. psittaci* infection.

**2)** To use the model to select recombinant proteins based on the MOMP sequence and produced by expression in an *E.coli* vector, which are able to cause a reduction in *C. psittaci* infection when formulated into a chlamydial vaccine.

**3)** To analyse the humoral response of individual sheep with respect to individual variable segments, and to test the protective capacity of antibodies produced against these regions after experimental abortion in sheep.

# CHAPTER 2

## MATERIALS AND METHODS

## 2.1. Animals

### 2.1.1. Pregnant ewes

Female Blackface x Swaledale sheep aged between 4-5 years were used in annual lambing experiments. All sheep were certified free from abortifacient *C. psittaci* under the Premium Sheep Health Scheme (Scotland).

The sheep were treated with Heptovac P (HAH Ltd) before transportation to Moredun Institute and wormed on arrival with anthelmintic. The ewes were isolated from other sheep throughout the duration of the experiments. Before challenging with live chlamydiae, the uninfected control group was separated from the rest of the experimental animals.

Oestrus was synchronised in the ewes with ('Veramix', Upjohn Ltd.) and after one full oestrus cycle the ewes were allowed to run with the tups.

### 2.1.2. Non-pregnant sheep

Greyface whether lambs aged 5 months old were used to produce serum for testing in *in vitro* and murine models. All lambs tested negative for chlamydial antibodies prior to exposure to *C. psittaci* as determined by immunoblotting of pre-experimental sera. None of these animals came into contact with animals from any other source whilst at Moredun.

### 2.1.3. Mice

***Active immunisation:*** Strain CBA/ ADRA were used aged 3-4 weeks old at primary vaccination. Blood samples taken from the lateral tail veins were tested for antibodies to *C. psittaci* before the start of each experiment and found to be negative by immunoblotting. Mice were assigned to groups by random allocation, with restrictions on both age and sex.

***Passive immunisation:*** Strain CBA/ADRA and strain CBA/CaOLaHsd (Harlan Olac Ltd.) were used aged 6-12 weeks old and between 15-20g in weight. Group allocation and blood sampling were carried out as for active immunisation.

## 2.2. Cells

### 2.2.1. Growth of inoculum

L929 cells, mouse fibroblasts were used to grow inoculum from stocks of egg-grown *C. psittaci*. L929 cells originated from normal areolar and adipose tissue from a 100 day old male C34/An mouse.

### 2.2.2. Titration of inoculum

BHK-21 cells , baby hamster kidney cells of fibroblastic origin were used to titrate inoculum.

### 2.2.3. Serum neutralisation assay

McCoy cells of fibroblastic origin, were used in *in vitro* serum neutralisation assays.

## 2.3. Chlamydial Inoculum

**Production:-** Egg grown abortifacient *C. psittaci* , strain S26/3 (5 passages in eggs) was used to infect L929 cells (mouse fibroblasts) in 225cm$^2$ tissue culture flasks. Complete RPMI was used as infection medium, containing antibiotics, 2% new born calf serum (NBCS) and 1µg/ml cycloheximide (Appendix 1). The inoculum was passed 2-6 times, using a "hot" passage technique and stationary incubation in tissue culture, before harvesting with glass beads and centrifuging at 12,000 rpm for 30 min at 6$^0$C. The pellet was resuspended in a small volume of cold PBS and homogenised in a glass homogeniser to break up the clumps. After recentrifuging, the pellet was washed again with cold PBS, recentrifuged and finally resuspended in CTM to 1/10 the original volume. The inoculum was aliquoted into Eppendorf tubes (1ml) before being frozen at minus 70$^0$C. Aliquots of chlamydial inoculum were titrated in BHK-

21 cells. The procedure was repeated for other batches of inoculum, including strain S95/3.

***Titration*:-** McCoy cells were grown in RPMI maintenance medium (Appendix 1) for three days at 37°C, 5% $CO_2$ in 225cm$^2$ tissue culture flasks. Once the cells had formed a confluent monolayer, the flask was rinsed with sterile PBS which had been pre-warmed to 37°C, and 2ml of a pre-warmed trypsin/versene (TV) mixture was added. Most of the TV was tipped off, leaving a thin covering over the cells. The flask was then incubated for 2-5 mins and sharply tapped to dislodge the cells. The volume in the flask was made up to 20ml with maintenance medium and a small sample (100µl) taken for assessment of the cell concentration. To do this, 100µl of cell suspension was diluted 1:1 with 0.04% trypan blue and the number of cells in both sides of a Neubauer chamber counted under the light microscope. The concentration of cells was adjusted to 2 x $10^5$ cells/ml. 1ml of cell suspension was pipetted into each "Trac" bottle (Sterilin) containing 1 x 16mm coverslip and the air bubbles underneath the coverslip removed by flicking the bottle. This prevented cells from growing on the underside of the coverslip. The "Tracs" were then incubated overnight at 37°C, 5% $CO_2$ and used the following day. 1ml of inoculum was added to each "Trac" at ten-fold dilutions and centrifuged at 2000x*g* for 30 min. The lids were loosened and the "Tracs" incubated at 37°C/5% $CO_2$ for 3 days. Coverslips were then fixed in methanol and stained for 20 min. in a 5% solution of Giemsa (BDH). After rinsing coverslips in water and dehydrating and clearing through graded concentrations of acetone-xylene mixtures, the coverslips were mounted in DPX on to microscope slides. The number of inclusions per coverslip was estimated by counting the whole coverslip at x200 magnification.

### 2.3.1. Sheep Inoculum

Challenge strains comprised S26/3 and S95/3, both of which were isolated from outbreaks of OEA in Scotland and grown in embryonated eggs through 6 and 2 passages, respectively. Strains were mixed in a 1:1 ratio, giving a final titre of 5 x $10^4$ ifu per ml in BHK-21 cells.

### 2.3.2. Mice Inoculum

Strain S26/3, isolated from an outbreak of OEA in Scotland was used in both active and passive immunisation experiments. Egg grown inoculum ($5^{th}$ passage) was grown in L929 cells for a further 6 passages before titration in BHK-21 cells, giving a titre of $1 \times 10^8$ ifu per ml in BHK-21 cells.

### 2.3.3. Serum Neutralisation Assay Inoculum

Egg grown inoculum, strain S26/3 ($5^{th}$ passage, as above) was passaged twice through L929 cells to give a titre in BHK-21 cells of $8 \times 10^6$ ifu per ml.

## 2.4. Inoculation procedures

### 2.4.1. Sheep

Challenge inoculum was held on ice to reduce loss of viability whilst the flock was injected. Each sheep received a 2ml dose given subcutaneously over the pre-clipped left shoulder. A separate needle was used for each animal. Negative control animals were left unchallenged.

### 2.4.2. Mice

Unless otherwise stated, all mice receiving inoculum were injected with a 1ml volume by the peritoneal route,. In some experiments, intravenous inoculation via the lateral veins was attempted.

All experiments involving passive immunisation of mice required preparation of inoculum mixtures to be injected intraperitoneally as follows:- Serum samples were defrosted and heat inactivated at 56°C for 30 mins. Chlamydial inoculum was defrosted and allowed to reach 37°C as was RPMI. With all the reagents at the appropriate temperature, serum samples were diluted to a working concentration of 1/2 in RPMI. Likewise, inoculum was diluted to a working concentration of $4 \times 10^6$ ifu/ml in RPMI ($2 \times 10^6$ ifu/ml in some cases). Diluted serum samples and diluted

inoculum were mixed in a ratio of 1:1 to give a final serum concentration of 1/4 and a final concentration of inoculum of 2 x $10^6$ ifu/ml (alternatively, 1 x$10^6$ ifu/ml) in each inoculum mixture. The inoculum mixtures were vortexed thoroughly and incubated at 37°C in a water bath for 45 mins with occasional mixing. After the incubation time was complete, the inoculum mixtures were placed on ice before injecting into mice. If several inoculum mixtures were used, preparation times were staggered to minimise the length of time each was kept on ice before injection into mice.

### 2.4.3. Serum Neutralisation Assays

Tissue culture grown chlamydial inoculum was thawed and allowed to reach 37°C in a water bath. Heat inactivated serum samples were pooled and also warmed to 37°C, before being added to an equal volume of diluted chlamydial inoculum (dilution factors recorded with appropriate chapter) and vortexed to mix. Inoculum mixtures were incubated at 37°C for 45 min. with occasional mixing, before inoculation of 1ml of mixture on to cell monolayers. After 2 x 30 min. periods of centrifugation at 2000x*g* in a bench top centrifuge, the infected cells were incubated in a 37°C incubator for 2h. Finally, the cell monolayers were washed with 3 changes of warmed PBS (37°C) and incubated for 3 days at 37°C/5% $CO_2$ in infection medium.

## 2.5. Antigen Production

### 2.5.1. ELISA

(Sarkosyl purified EBs were kindly prepared for use in the indirect ELISA by Judith Machell using the methodology of Ian E. Anderson).

Tissue-culture grown *C. psittaci* was harvested from flasks using sterile glass beads and centrifuged at 12K for 30 min. at 6°C. The pellet was resuspended in PBS, homogenised with a glass homogeniser and sonicated for 2 x 30 sec. The sample was then treated with 1% sarkosyl and incubated at 37°C with constant stirring. After 2h, the sarkosyl treated EBs were recentrifuged, washed with 3 changes of PBS

and stored in PBS at -20°C if not required immediately. The concentration of MOMP in the sample was estimated by densitometric analysis of immunoblots. The sarkosyl treated EBs (2.2mg) were then suspended in 0.1M PBE (4.66ml) and 1.0M sarkosyl added (340µl). *The EBs were incubated for 1h at 37°C, during which time the sample was vortexed and sonicated (4x5sec) every 15 min. After 1h, the EBs were dispensed into 5x1ml amounts in eppendorphs and pelleted on a microfuge (13000rpm/30 min)**. The supernatants were discarded*** and each pellet was resuspended in 0.1M PBE (0.624ml). At this point, 1.0M sarkosyl (170µl), followed by 0.1M octyl glucoside (1.71ml) was added and the sample treated as before (* to ***). The pellet was then resuspended in 0.1M PBE (0.956ml), transferred to a clean vessel and 1.0M sarkosyl added (170µl) with 1.0M DTT (50µl). Steps * to ** were repeated and the final supernatant collected for use as ELISA antigen, enriched for the MOMP fraction.

### 2.5.2. Vaccine antigens

#### 2.5.2.1. Native chlamydial antigen

Tissue culture grown chlamydiae were produced as in Section 2.3. and formulated into vaccines as described in Section 2.5.3.

#### 2.5.2.2. Purified MOMP

Chlamydiae were grown in tissue culture, harvested using sterile glass beads and the cells disrupted using a Jencons-glass-Teflon homogeniser. The culture was clarified by centrifugation (1500*g*/5mins/4°C) and then pelleted through a 10ml density cushion of 30% (v/v) Urografin in Tris KCl (20mM Tris KCl pH 7.5 + 150 mM KCl) by centrifugation (53000*g*/45mins/4°C). The crude chlamydial pellet was resuspended in a small amount of Tris KCl (1ml) and homogenised to disrupt aggregates before layering on to a 30%-60% Urografin continuous density gradient. Chlamydial EBs and RBs were ultracentrifuged (53000*g*/2h/4°C) to purify. Two opalescent bands which appeared were collected using a syringe and diluted in Tris

KCl. The samples were repelleted by centrifugation (53000*g*/45mins/4°C) and resuspended in Tris KCl (100-500µl) using a homogeniser.

### 2.5.2.3. Recombinant antigens

Recombinant MOMP constructs were kindly gifted by Dr Alan Herring (Department of Biochemistry, Moredun Research Institute). A summary of the expression of recombinant constructs of MOMP in standard *Escherichia coli* systems is given below (A. J. Herring, personal communication).

The initial MOMP expression contructs were fusions of the first few amino acids (AAs) of β-galactosidase with either full length mature MOMP, 367 AAs (fMOMP-pD) or the C-terminal 288 AAs (fMOMP-pC). In the present study, construct fMOMP-pD was used. The vector used in both fMOMP constructs was pUC-8 and in each case, the leader sequence was removed before fusion. As a result, the synthesised MOMP was not translocated across the inner membrane of *E.coli*. Fusion MOMP (fMOMP) was therefore spatially restricted to the cytoplasmic region where large amounts resulted in the formation of insoluble inclusion bodies.

The second type of recombinant MOMP investigated was designed in order to overcome the potential problem of MOMP forming insoluble cytoplasmic inclusions. As such, translation was designed to start at the natural initiator codon i.e. the first methionine. The inserted gene sequence thus included the signal sequence of MOMP (16 amino acids). With this arrangement, it was hoped that MOMP would be translocated to the outer membrane and assume a native conformation. Hence, the acronym, membrane MOMP (mMOMP). This construct was made in the pET-22 vector. Indications that MOMP was indeed present in the outer membrane came from cell fractionation experiments and immunoblot analysis with and without disulphide bond reduction. In contrast to fMOMP, mMOMP was found to be highly cross-linked resembling the structure of native MOMP. However, both N-terminal sequencing and size analysis revealed that signal peptide removal did not occur. The

vaccine preparation of mMOMP contained a suspension of detergent-extracted outer membranes in which MOMP was the most abundant protein.

A further two forms of recombinant MOMP were examined as vaccine antigens. Previously, the whole sequence coding for the native MOMP protein was placed downstream of the vector pelB leader sequence using a Stu-I site introduced by PCR mutagenesis and the natural EcoRI site downstream of MOMP. MOMP expression in this contruct was found to be highly unpredictable and again signal peptide removal did not occur. However, a small amount of correctly processed MOMP was observed in the newly transformed cells without induction. This observation prompted design of the first of these constructs, in which a Bgl-II/EcoRI fragment from the pET-22b construct was cloned into the pUC-19 vector to give a bi-cistronic system in which pelB MOMP was co-expressed with the N terminal part of the lac Z gene. Tandem operator regions, one from pUC-19 and one from pET-22b, gave tight control by the lac $I^q$ repressor. This construct was termed mMOMP19. Whilst incomplete, evidence suggests that this construct is only translocated as far as the periplasmic space and is not incorporated into the outer cell wall.

The final construct in pET-22b was designed to produce large amounts of MOMP peptide for renaturation experiments. It consisted of the coding region for the C-terminal 351 AAs of MOMP cloned into the Nde-I and EcoRI sites of pET22b using an Nde-I site in the MOMP coding region introduced by PCR and the natural downstream EcoRI site. On induction, the construct produces a protein lacking the first 16 AAs of MOMP. This truncated form was thus termed tMOMP.

In addition, variable segments of MOMP were expressed as novel pGEX fusion peptides expressed in *E.coli* with the enzyme glutathione GST. Purification of VS1 and 2 produced in this manner is described below.

***VS1:*** **Growth in culture**:- Three colonies expressing VS1 were grown from an agar plate. Each colony was grown in 4ml L-broth + ampicillin. At mid-log phase the colonies were induced by the addition of IPTG to 1mM and grown for a further 3 h. 0.5ml was removed from each flask, centrifuged and washed in PBS. The pellets were then resuspended in 100µl of 2x sample buffer. 10µl of each sample was run on an SDS-PAGE mini-gel to check for induction by staining.

Choosing the best sample, a 10ml culture was grown during the day to mid-log phase. This was kept at 4°C over-night. The following day 8ml of culture was centrifuged and used to inoculate 200ml of culture medium (L-broth + ampicillin 80µg/ml). The culture was allowed to grow for approximately 2h, until the broth reached an optical density of between 0.6-0.8nm. 100mM IPTG was then added to induce production of VS1 by the *E.coli* vector. After 3h further growth, the culture was removed from the incubator, centrifuged at 5K for 15 min. in 1xPBS (approximately 2x volume) to wash the pellet and then stored as a pellet at 4°C overnight. The following day, the pellet was resuspended in PBS and purified as described below.

**Purification of VS1 by glutathione sepharose 4B gel:**-Glutathione Sepharose 4B is designed for rapid single step purification of recombinant derivatives of glutathione S-transferase, other glutathione S-transferases or glutathione dependent proteins. Variable segment 1 (VS1) of *C. psittaci* (abortifacient) was expressed as a GST construct in *E.coli* as described above. Purification of VS1 was carried out on a GST-sephadex column as follows:-

The gel was packed into a chromatography column and washed with 5-10 bed volumes of PBS (containing 150 mM Na Cl, 16mM $Na_2HPO_4$, 4mM $NaH_2PO_4$, pH 7.3) to remove the preservative. The gel bed was equilibrated with 3 bed volumes PBS + 1% Triton X-100. The sample was then applied to the column and the eluent discarded. The column was washed again with 2x bed volumes of PBS. The bound

material was then eluted with 5 bed volumes of elution buffer (5mM Glutathione in 50 mM Tris-HCl pH 8.0) and the fractions collected (300µl, 1M Tris-HCl + 300µl, 100mM Glutathione in 6ml DW). The fraction collector was set at flow rate 7, Time, 040 (4 min), x1 (small tubes). The flow rate was turned up to maximum when packing the gel. Fractions were examined for VS1 content by SDS-PAGE. The specificity of VS1 was determined by immunoblotting using a GST control. Densitometry of SDS-PAGE gels was used to quantify the VS1 preparation.

The protocol described above for growth and purification of VS1 was repeated to produce the VS2-GST construct and GST only, as a control.

**Calculation of Variable Segment Concentrations from Densitometry Curves:-**

**BSA Standard 1**

Stock solution = 0.2 mg/ml; working dilution = 0.1 mg/ml

Loaded on to SDS page gel at 1.0 µg and 0.5 µg

| | | |
|---|---|---|
| Scan Number 3 | Peak 1 = 829 | Peak 5 = 1488 |
| | Peak 2 = 445 | Peak 6 = 882 |
| | Peak 3 = 500 | Peak 7 = 2359 |
| | Peak 4 = 959 | |

Sum of peaks = 7462 units, in 1.5 µg. Therefore, 1 unit = 1.5/7462 µg.

cont'd

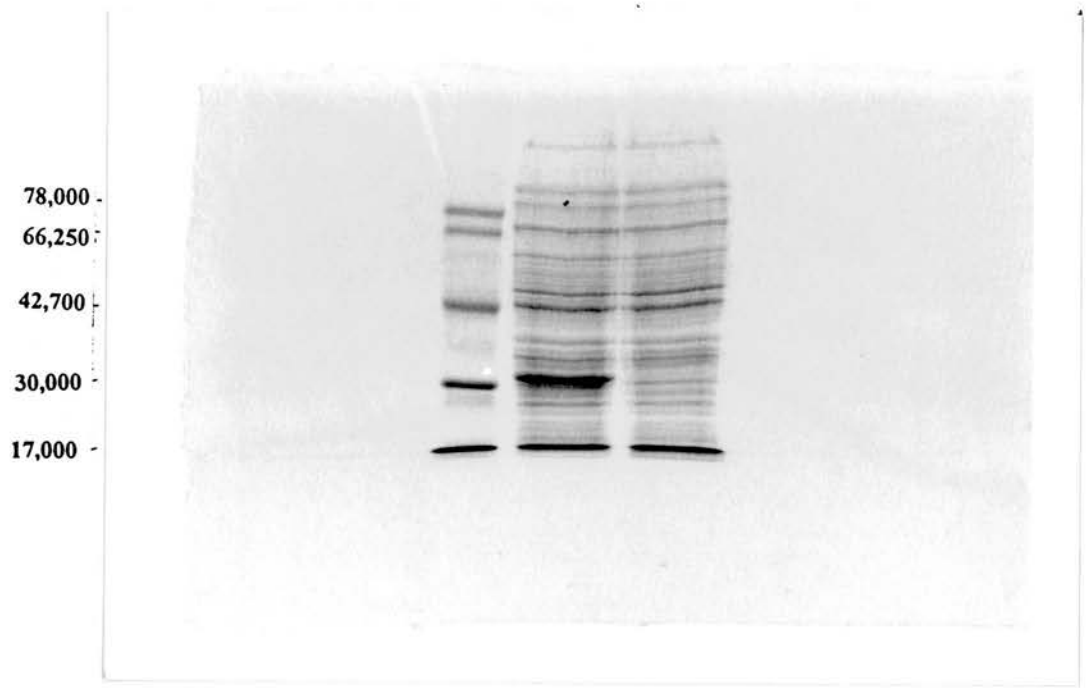


**Fig 2.1.** SDS PAGE gel of induced and uninduced cultures of transformed *E.coli* - VS1-containing fractions eluted from GST-Sephadex column (arrow indicates position of fusion protein)

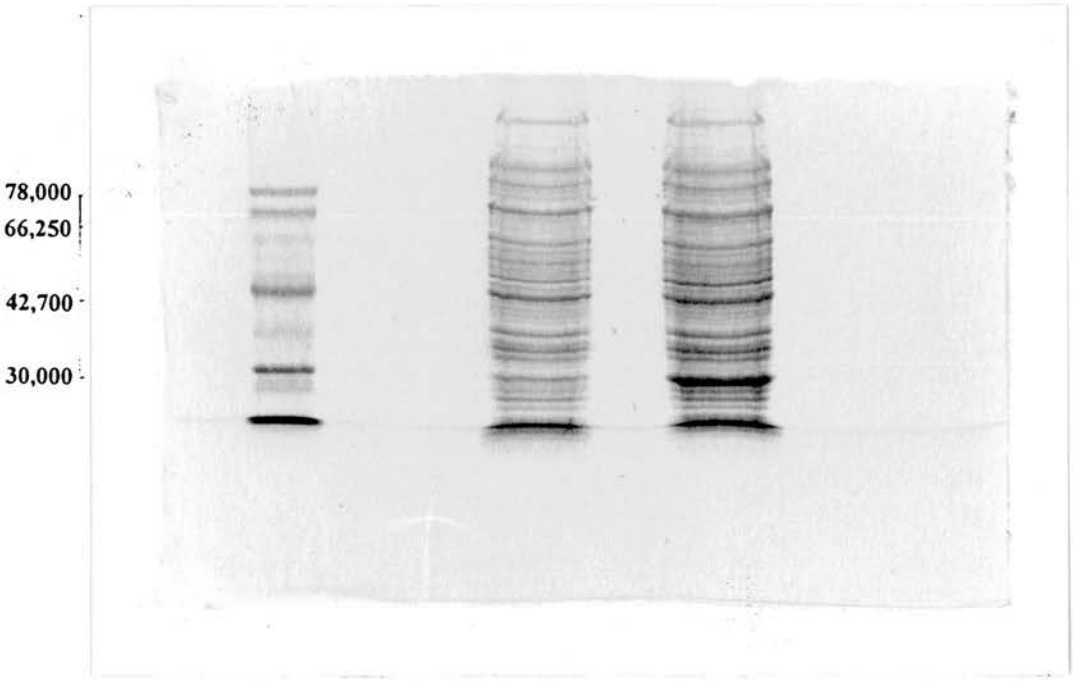


**Fig 2.2.** SDS PAGE gel of induced and uninduced cultures of transformed *E.coli* - VS2-containing fractions eluted from GST-Sephadex column (arrow indicates position of fusion protein)

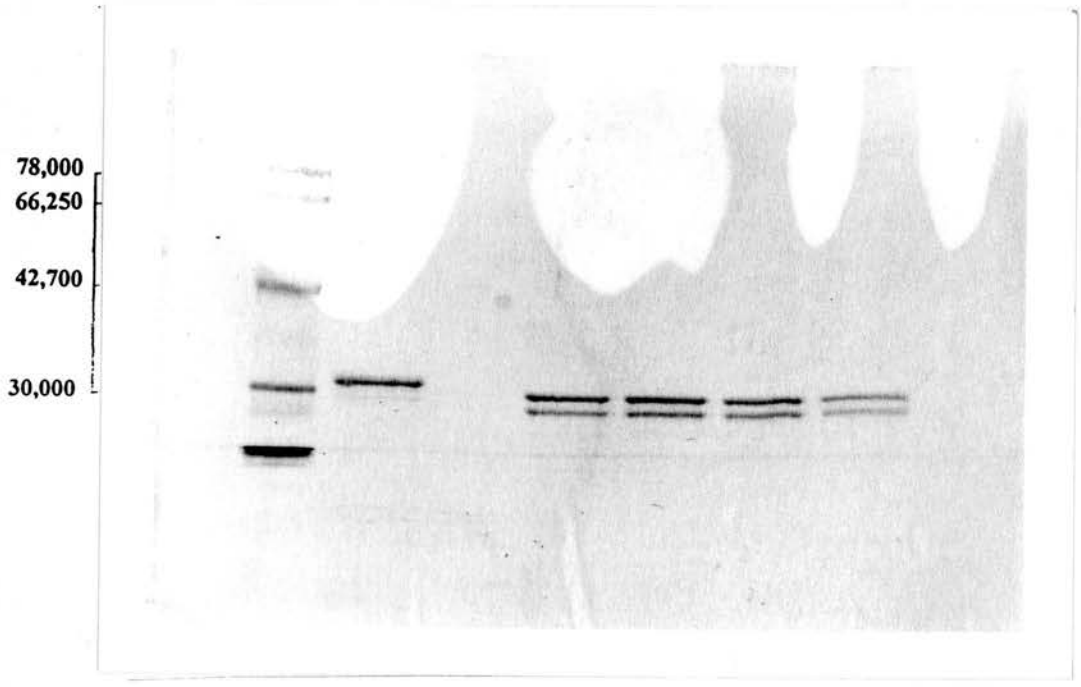


**Fig 2.3.** SDS PAGE gel of purified VS2-containing fractions eluted from GST-Sephadex column

cont'd

**GST Control**

Loaded on to SDS PAGE gel as 2.5 µl

Scan Number 5 Peak 1 = 6108 Peak 2 = 10203

Sum of peaks = 16311 units, in 2.5 µl.

Therefore, 16311x 1.5/7462 µg = 3.3 µg in 2.5 µl

GST concentration = 1.3 µg/µl

**VS2**

Loaded on to SDS PAGE gel as 1.0 µl

Scan Number 4 Peak 1 = 6558 Peak 2 = 3999

Sum of peaks = 10557 units, in 1.0 µl.

Therefore, 10557 x 1.5/7462 µg = 2.1 µg in 1.0 µl

VS2 concentration = 2.1 µg/µl

**BSA Standard 2**

Stock solution = 0.2 mg/ml; working dilution = 0.1 mg/ml

Loaded on to SDS page gel at 1.0 µg and 0.5 µg

Scan Number 1 Peak 1 = ----- Peak 4 = 2636

Peak 2 = 903 Peak 5 = 1412

Peak 3 = 1535 Peak 6 = 1241

Sum of peaks = 7727units, in 1.5 µg. Therefore, 1 unit = 1.5/7727 µg.

**VS1**

Loaded on to SDS PAGE gel as 2.5 µl

Scan Number 2 Peak 1 = 8263 Peak 2 = 7198

Sum of peaks = 15461 units, in 2.5 µl.

Therefore, 15461 x 1.5/7727 µg = 3.0 µg in 2.5 µl

VS1 concentration = 1.2 µg/µl

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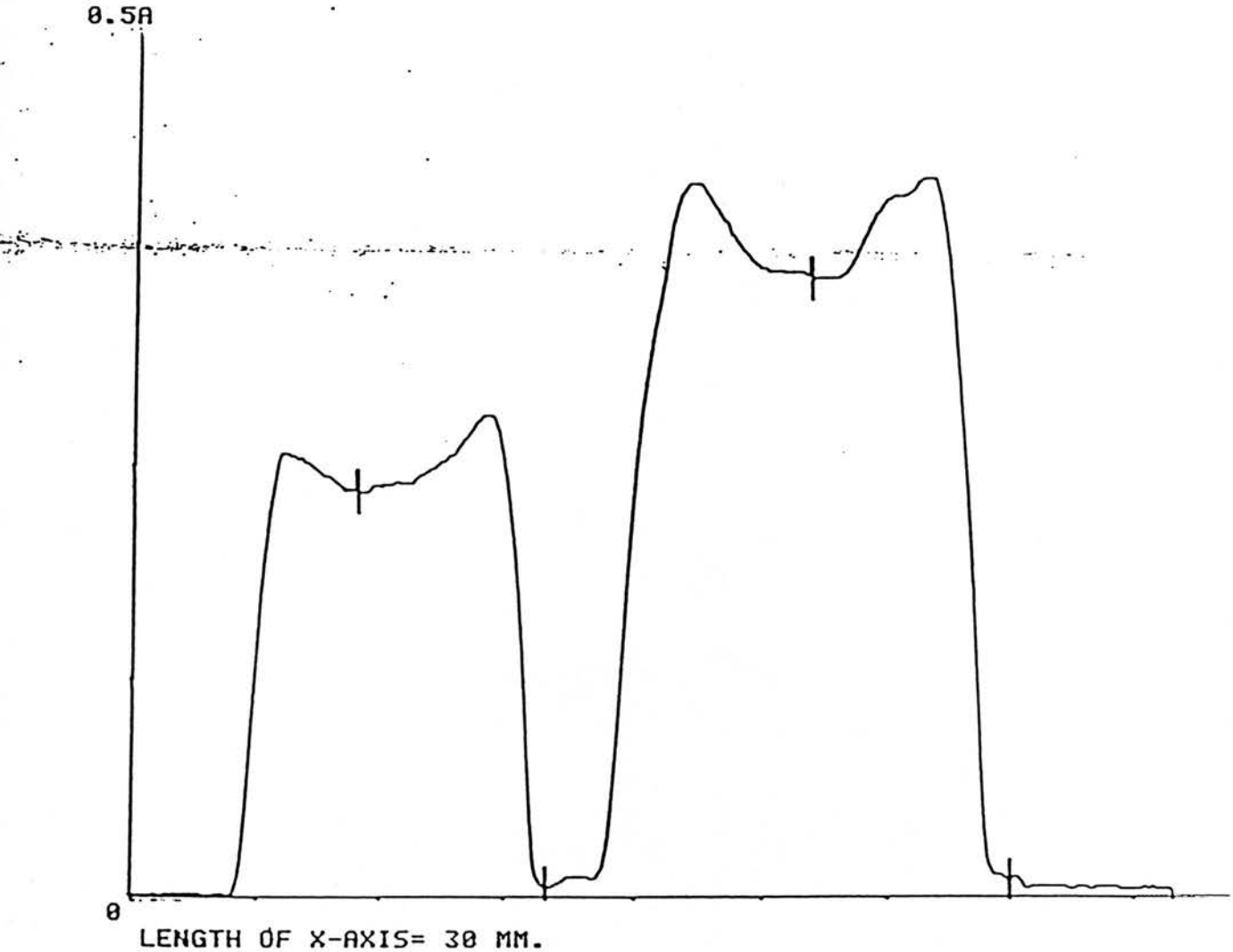


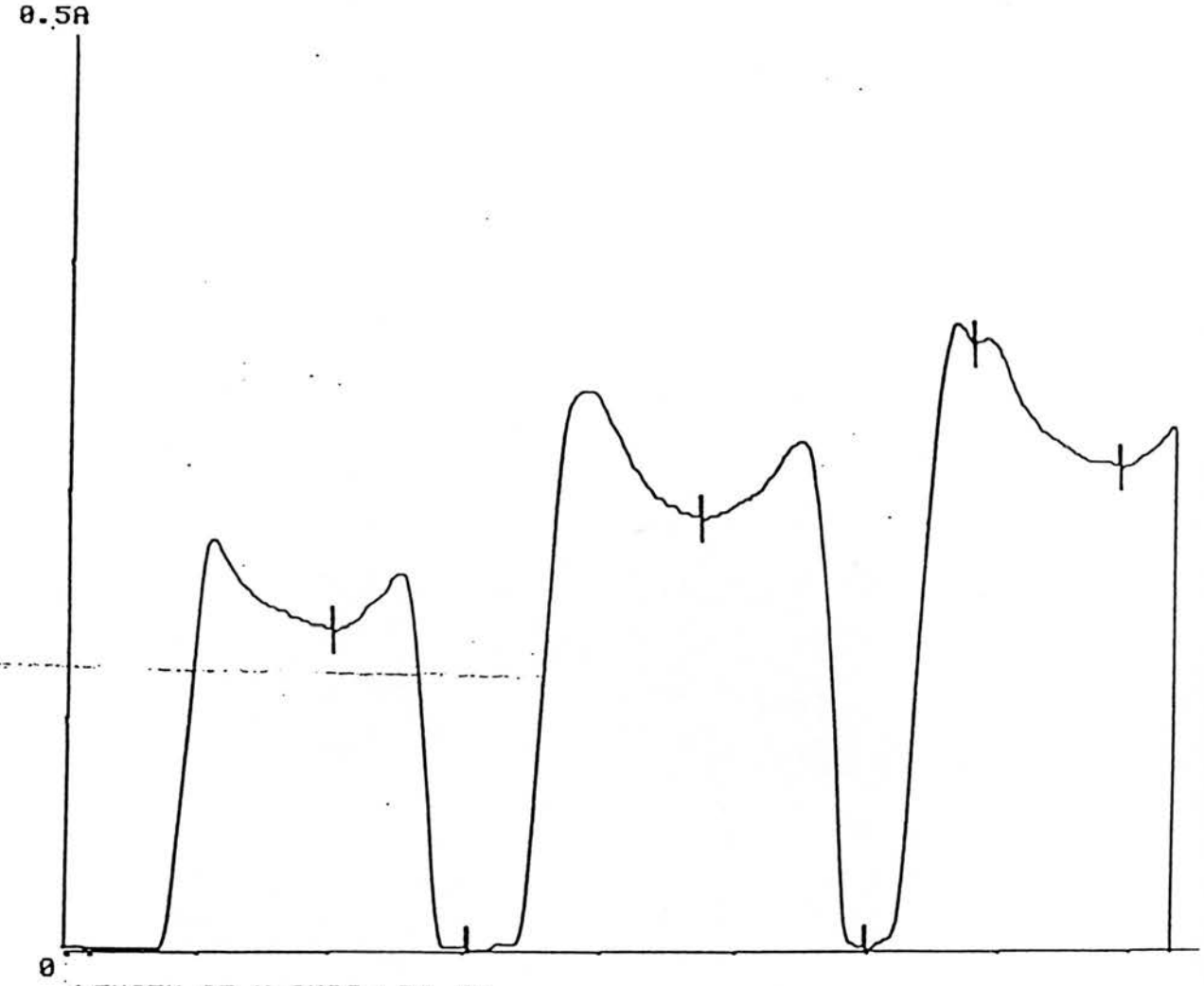

LENGTH OF X-AXIS= 30 MM.
TOT. INTEGRAL= 48019

| PEAK | POSITION | REL% | INTEGRAL | PEAK | POSITION | REL% | INTEGRAL |
|---|---|---|---|---|---|---|---|
| 1 | 3.65 | 12.71 | 6108 | 2 | 8.55 | 21.24 | 10203 |
| 3 | 13.25 | 33.68 | 16174 | 4 | 18.85 | 31.9 | 15320 |
| 5 | 21.15 | 0.44 | 214 | | | | |

LINEAR SCAN RUNNING...

SCAN NUMBER= 4

SCAN LENGTH= 25 MM. APERTURE WIDTH= 0.1 MM.




LENGTH OF X-AXIS= 30 MM.
TOT. INTEGRAL= 45377

| PEAK | POSITION | REL% | INTEGRAL | PEAK | POSITION | REL% | INTEGRAL |
|---|---|---|---|---|---|---|---|
| 1 | 3.35 | 14.45 | 6558 | 2 | 7.6 | 8.81 | 3999 |
| 3 | 11.6 | 21.06 | 9560 | 4 | 16.45 | 16.84 | 7643 |
| 5 | 19.95 | 8.61 | 3910 | 6 | 20.65 | 22.2 | 10076 |
| 7 | 24.95 | 8.0 | 3631 | | | | |

LINEAR SCAN RUNNING...

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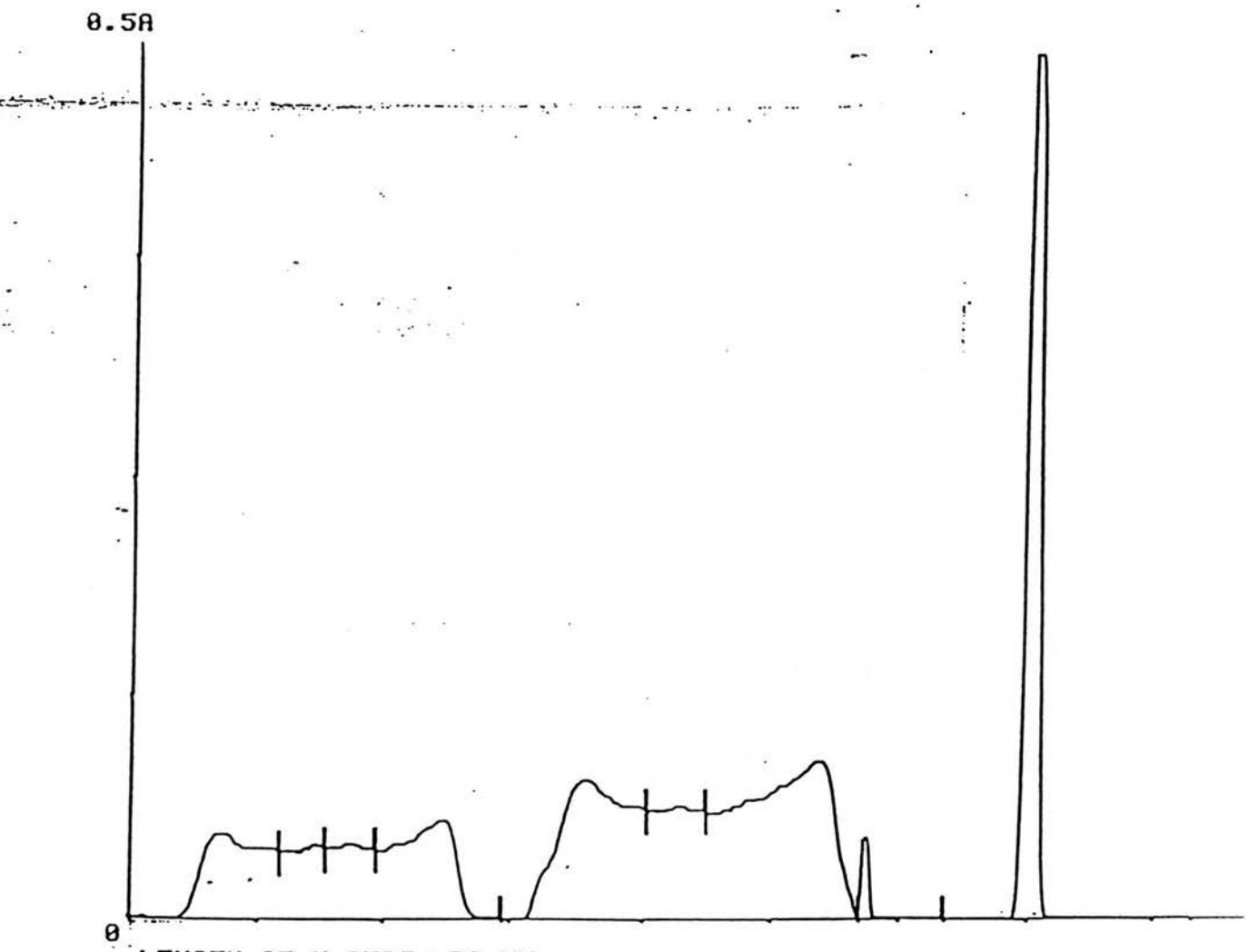
SCAN LENGTH= 25 MM. APERTURE WIDTH= 0.1 MM.

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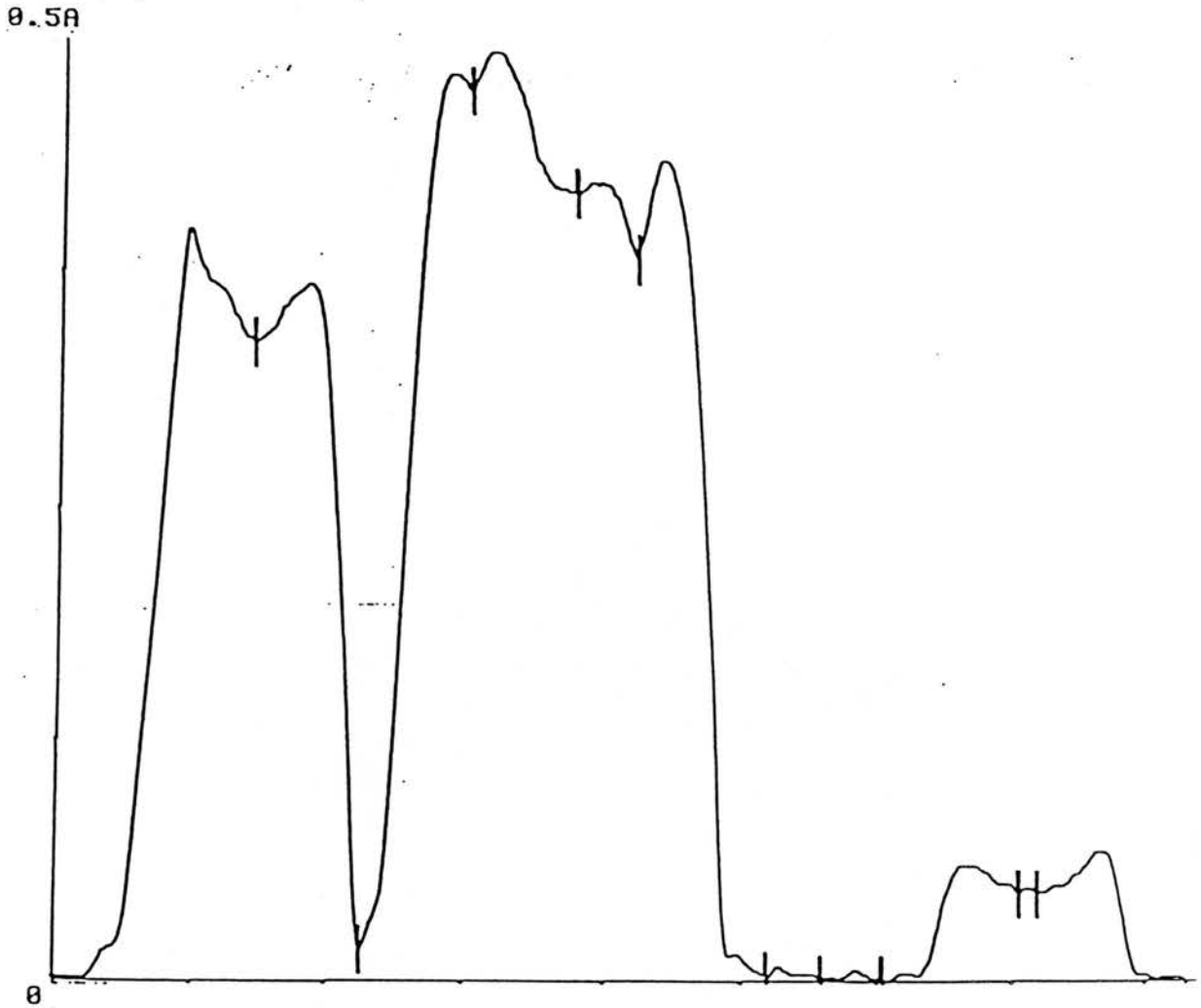
TOT. INTEGRAL= 9820

| PEAK | POSITION | REL% | INTEGRAL | PEAK | POSITION | REL% | INTEGRAL |
|---|---|---|---|---|---|---|---|
| 1 | 2.2 | 9.19 | 829 | 2 | 4.45 | 4.93 | 445 |
| 3 | 5.25 | 5.54 | 500 | 4 | 7.45 | 10.63 | 959 |
| 5 | 10.75 | 16.49 | 1488 | 6 | 12.9 | 9.77 | 882 |
| 7 | 16.15 | 26.15 | 2359 | 8 | 17.3 | 0.77 | 70 |
| 9 | 21.1 | 16.49 | 1488 | | | | |

LINEAR SCAN RUNNING...

SCAN NUMBER= 2

SCAN LENGTH= 25 MM. APERTURE WIDTH= 0.1 MM.




LENGTH OF X-AXIS= 30 MM.
TOT. INTEGRAL= 46892

| PEAK | POSITION | REL% | INTEGRAL | PEAK | POSITION | REL% | INTEGRAL |
|---|---|---|---|---|---|---|---|
| 1 | 2.85 | 17.62 | 8263 | 2 | 5.55 | 15.35 | 7198 |
| 3 | 8.6 | 13.26 | 6218 | 4 | 9.45 | 22.19 | 10409 |
| 5 | 11.55 | 12.02 | 5638 | 6 | 13.1 | 14.5 | 6803 |
| 7 | 15.9 | 0.07 | 33 | 8 | 17.6 | 0.04 | 20 |
| 9 | 19.95 | 2.1 | 986 | 10 | 21.35 | 0.4 | 192 |
| 11 | 22.95 | 2.41 | 1132 | | | | |

MODE: %= 0, ABSORBANCE
MAX. ABSORBANCE: .5
PEAK RESULTS: (YES=1,NO=0) 1
PEAK/TROUGH THRESHOLDS: HEIGHT= WIDTH= NOISE=
BACKGROUND CORR.: (YES=1,NO=0) 0
AUTO ZERO: (YES=1,NO=0) 1

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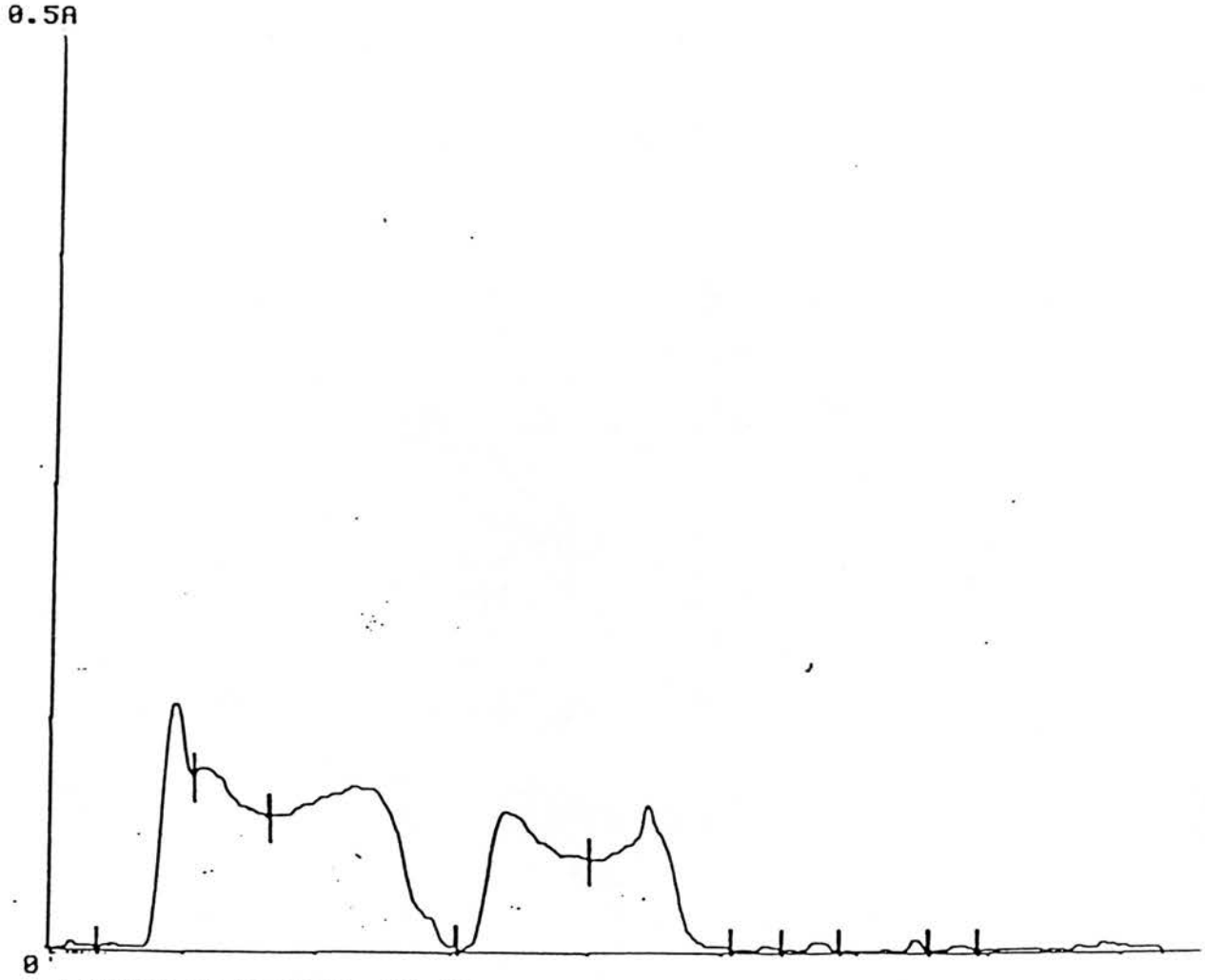


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TOT. INTEGRAL= 7928

| PEAK | POSITION | REL% | INTEGRAL | PEAK | POSITION | REL% | INTEGRAL |
|---|---|---|---|---|---|---|---|
| 1 | 0.55 | 0.22 | 18 | 2 | 2.85 | 11.39 | 903 |
| 3 | 3.5 | 19.36 | 1535 | 4 | 6.95 | 33.24 | 2636 |
| 5 | 10.25 | 17.81 | 1412 | 6 | 13.4 | 15.65 | 1241 |
| 7 | 16.05 | 0.25 | 20 | 8 | 17.2 | 0.37 | 30 |
| 9 | 19.4 | 0.27 | 22 | 10 | 20.4 | 0.29 | 23 |

### 2.5.3. Vaccine formulations

Reagents:- i) Montanide ISA 50, Lot 2011 Seppic/ Marcol Arlacel A

ii) Alhydrogel (Superfos).

iii) Glutaraldehyde (BDH), Product N°. UN2810, Concentration 50%.

iv) Thimerosal, stock solution 1% (w/v) in distilled water.

Method:-

Each vaccine was formulated by slowly mixing equal volumes of aqueous and adjuvant portions using an Ystral homogeniser. Smaller quantities were emulsified by the "syringe to syringe" method. The adjuvant phase comprised: Marcol Arlacel A or Montanide ISA 50 as 50% of the final volume. The aqueous phase comprised: Alhydrogel at 25% final volume; Antigen at 50-100μg/ml final concentration; Glutaraldehyde at 0.1% final volume; Thimerosal (1%) at 0.01% final volume and PBS to make up the volume to 50% of the final volume.

For 60ml of vaccine: Antigen was made up to 6ml with Tris buffer, 6μl of neat glutaraldehyde added and the mixture held at room temperature for 15 mins. 24ml of Alhydrogel (Tris) was added, followed by 60μl of thimerosal. Adjuvant was dispensed into 4x7.5ml aliquots. Whilst holding over ice, 3ml of aqueous solution was slowly added to 1 aliquot of adjuvant as the mixture was homogenised with an Ystral homogeniser for 30 secs. The remaining 2ml were added in a similar fashion and the whole emulsification process repeated with the remaining 3 aliquots of adjuvant. The homogenised aliquots were pooled in a 100ml duran bottle and homogenised for a further 30 secs. Vaccine aliquots were stored at 4°C and samples submitted for bacteriological screening.

## 2.6. ELISA Protocols

### 2.6.1. Indirect serum ELISA

Sarkosyl purified chlamydial antigen (Section 2.5.1.) was ultrasonicated (2 x 5 sec bursts), then diluted in $CO_3/HCO_2$ buffer to a concentration of 20 μg/ml. A fresh 96-

well plate (Nunc) was taken and 100 µl of diluted antigen (2µg) was added to each well in alternate rows. The rest of the plate was then filled with 100 µl $CO_3/HCO_2$ buffer as control wells. The plate was sealed and stored at 4°C overnight.

The following day, the plate sealer was removed and the plate contents vigorously ejected. The plate was washed three times with PBS/Tween and blotted dry. 120µl periodate solution was added to each well and the plate resealed and placed on a plate shaker for 10 mins, followed by a 20 min stationary incubation at 37°C. After washing the plates as before, 200µl of 10% horse serum in PBS/Tween was added to all wells. The plate was again sealed, shaken for 10 mins and incubated for 20 mins.

After washing, 120µl of 0.5% glutaraldehyde in PBS/Tween was added and the plate placed in the refrigerator at 4°C for 25 mins. Some plates were stored at 4°C in sealed bags at this point for future use. To continue the assay, test samples were applied at a dilution of 1/800 in PBS/Tween in quadruplicate in cubic formation. The plate was sealed and incubated as before. After 30 mins, the plate was washed and 100µl of conjugated antibody added to each well. For detection of ovine antibodies, donkey anti-sheep IgG conjugated to horseradish peroxidase SAPU) was used at a dilution of 1/2000. Murine antibodies were detected with goat anti-mouse gamma globulin conjugated to horseradish peroxidase (SAPU), diluted 1/500 in PBS/Tween. After incubation as before, the plate was washed and 100µl substrate (OPD; Sigma Chemicals) added to each well. The plate was left in darkness for 10 mins until the colour of the positive control had developed sufficiently. The reaction was stopped by the addition of 50µl 2.3M $H_2SO_4$. Absorbencies were read on an ELISA reader (Titertek™ ) at 492 nm.

### 2.6.2. Variable segment capture ELISA

VS1 peptide was produced by the author as described in section 2.5.2.3. The ELISA plate was coated with 0.2µg/well of antigen diluted in PBS/Tween and a GST preparation was used as a control by coating adjacent wells. The plates were prepared the day before use, covered and kept at 4°C overnight. The following day,

the plate was washed and blocked with 200µl/well of 10% horse serum and incubated as above. After washing the plate, serum samples were diluted 1/1000 in PBS/Tween and 100µl of each sample pipetted into adjacent wells in a rectangular formation. The ELISA procedure now continued as above. VS2 was also prepared and used to coat ELISA plates to detect antibodies specific for the second variable segment of MOMP.

### 2.6.3. IgG isotyping ELISA

The indirect serum ELISA (section 2.6.1.) was used as the basis for this assay. However, modifications were made and the system optimised using immune and non-immune sheep sera. Plates were coated with sarkosyl antigen as before and serum samples applied at a dilution of 1/100 in 1% horse serum. An additional step involving the application of mouse α-sheep IgG1 or IgG2 diluted 1/500 (Serotec) was incorporated, before detection of antibodies by goat α- mouse gamma globulin conjugated to horseradish peroxidase (SAPU), diluted to 1/500.

### 2.6.4. LPS-ELISA

An LPS-ELISA was developed at the Moredun Institute to analyse chlamydial infection of sheep placental material (Jones, unpublished) and adapted for use with mouse samples (this thesis). The ELISA plates were coated with 100µl /well of raw ascitic fluid for Mabs 13/4 and 13/5, mixed in a ratio of 1:1 and used at a final dilution of 1/2500 in $CO_3/HCO_2$ buffer. The plate was sealed and stored for at least 12h at 4°C. The following day, the plate was washed with PBS/Tween three times and the wells blocked with 200µl of 10% Marvel in $CO_3/HCO_2$ buffer. The plate was sealed and placed on a shaker for 10 mins before incubation at 37°C for 20 mins. This washing and incubating procedure was carried out after each step. Samples were then applied to duplicate wells. Processed mouse organs were used at a dilution of 1/5 or 1/10, whilst peritoneal exudates were used at 1/2 in PBS/Tween. Positive control samples comprised LPS from *Salmonella minnesota.* Detection of chlamydial LPS was achieved by the addition of 100µl Mab 13/4 conjugated to HRP

at a dilution particular to each batch and development of a coloured product using the substrate OPD. As before, 50µl/well, 2.3M $H_2SO_4$ was used to stop the reaction and absorbencies were read on an ELISA reader at 492nm.

### 2.6.5. Competitive ELISA

IEA-antigen (section 2.5.1) was diluted 1/320 in 0.05M $CO_3/HCO_3$. An ELISA plate (Greiner F) was coated with 100µl diluted antigen to all wells except those in the first column. The plate was sealed and placed at 4°C overnight. The plates were washed according to standard practise and non-specific binding sites blocked by the addition of 200µl 10% horse serum in PBS/Tween and incubated for 1h at 37°C. 1ml of positive and negative control sera were prepared at a dilution of 1/20 in PBS/Tween containing 1% horse serum. Doubling dilutions of each test sample were similarly prepared between 1/10 and 1/80 dilutions. After washing, 100µl of each test sample was added to duplicate wells. 8 wells were used for each control sample. The plate was incubated for 1h at 37°C and then without aspirating the sera, 10µl Mab 4/11 at a dilution of 1/800 in 1% horse serum was added to each well. The plate was incubated for a further 1h at 37°C. After washing the plate, 100µl of α-mouse gamma globulin conjugated to HRP (SAPU) was added to each well. The plate was incubated for 1h at 37°C. Finally, the plate was washed and 100µl OPD substrate added per well. The plate was incubated in the dark for approximately 20mins and then the reaction stopped by adding 50µl of 2.3M sulphuric acid. The optical densities were read at 492 nm using a Titertek Multiscan reader (ICN Flow Ltd). Percentage inhibition was calculated for each test sample as follows:-

$$\frac{\textit{mean absorbance negative control - mean absorbance test sample}}{\textit{mean absorbance negative control - mean absorbance positive control}} \times 100$$

### 2.7. Serum Samples

Sheep were bled from the jugular vein into 10ml vacutainers. Samples were allowed to clot overnight at room temperature and then centrifuged at 3000 rpm and the

removed with a Pasteur pipette. Serum samples were aliquoted into medicine bottles and stored upright at -20°C. Mice were bled from the lateral tail veins using a Gilson pipette to collect blood from a small incision. Samples were collected in 1.5 ml eppendorfs and treated as above.

### 2.7.1. Ammonium sulphate precipitation of polyclonal sheep serum

Sheep immune serum taken from a pool of ewes after aborting, was used to provide antibodies against *C. psittaci* for use in the immunoperoxidase technique described above. A saturated solution of ammonium sulphate was prepared by adding 76.1g of ammonium sulphate to 100ml of distilled water. The amount of ammonium sulphate solution to be added to 10ml of positive serum was calculated as follows in order to arrive at a final concentration of 45% saturation, once the reagents were mixed:-

$$Volume\ of\ NH_4(SO_4)_2 = \frac{volume\ of\ sample \times final\ concentration}{1 - final\ concentration}$$

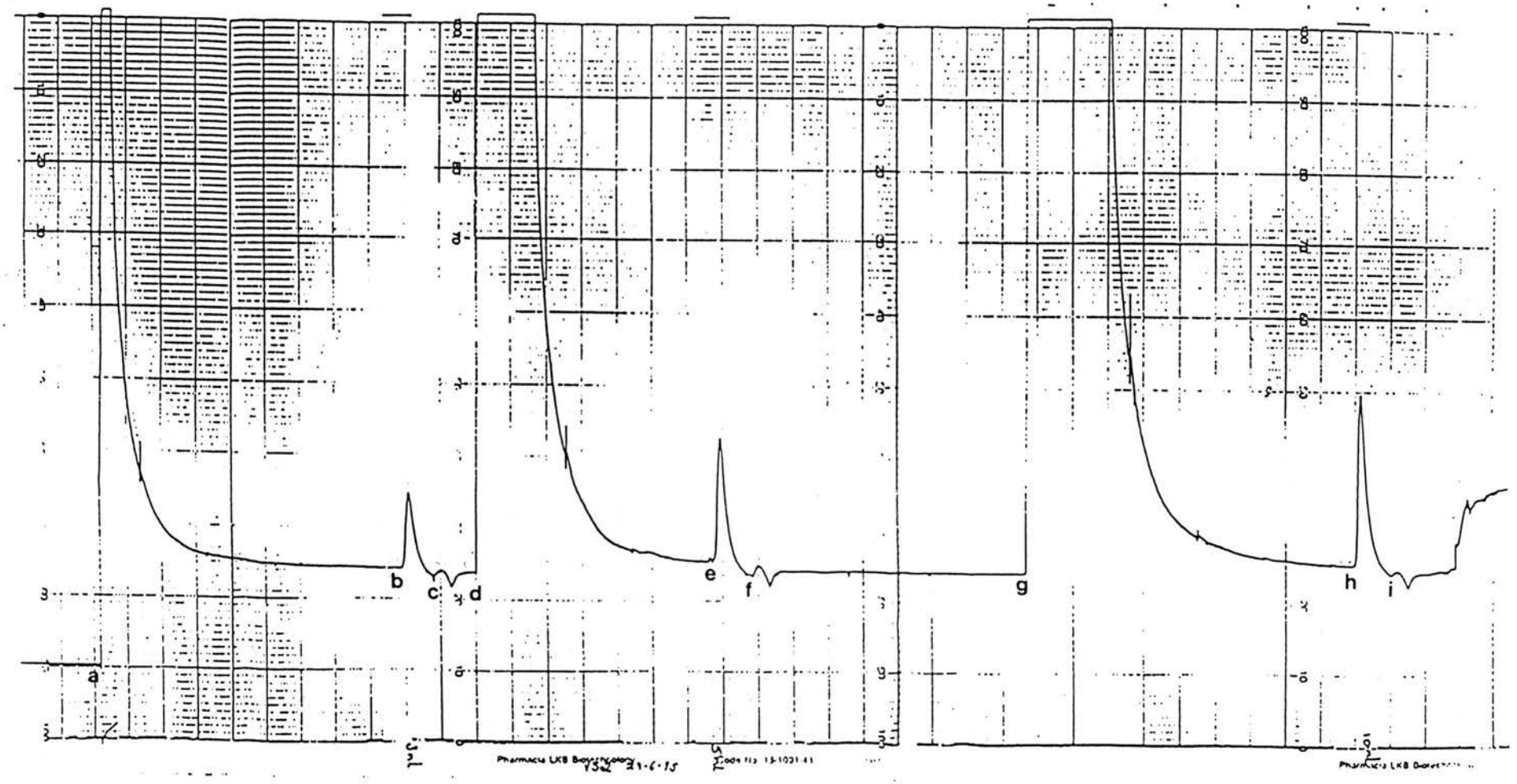
Precipitation of fibrinogen and transferrin was avoided by using a 45% rather than 50% saturated solution of ammonium sulphate. The ammonium sulphate solution was added in a dropwise fashion over ice (approximately 2h) using a burette attached to a peristaltic pump. The mixture was stirred constantly on a magnetic stirrer. After all the ammonium sulphate solution had been added, the mixture was allowed to stand at 4°C overnight. The following day, the solution was centrifuged in a bench top centrifuge at 2000x*g* for 1h. The resulting pellet was carefully resuspended in PBS to 0.2 x original volume of serum with minimal agitation. Finally, the solution of antibodies was dialysed against 3 changes of PBS at 4°C overnight. Titration to determine the optimal working dilution for the immunoperoxidase technique was performed on mouse tissue.

### 2.7.2. Affinity purification of antibodies against VS1 and VS2

VS1 was coupled to cyanogen-bromide sepharose beads and prepared as an affinity column described below. Serum from a standard pool of sheep immune serum was

poured down the column and the eluate quantified for total protein by Pierces BCA method. The eluate was then dialysed against PBS until a concentration of approximately half that found in the original serum sample was obtained. The same procedure was repeated for VS2. This time, however, the fall through from the VS1 affinity column was used for the VS2 column.

Affinity Column Purification of VS2 using Cyanogen bromide Sepharose



***Coupling VS1 and VS2 to cyanogen-bromide sepharose[r] 4B:*** Cyanogen-bromide activated sepharose[r] 4B (CNBr-sepharose) (Pharmacia Biotech) is a preactivated gel for immobilisation of ligands containing primary amines. The peptides VS1 and VS2 were coupled to the gel using the following protocol:- 1g of freeze-dried CNBr-sepharose was weighed out into a universal bottle. Approximately 10ml 1mM HCl was added and the gel allowed to swell for 1hour on a spiral mixer. At this point, 0.5mg VS1 (or VS2) was measured into a centricon-10 filter and made up to 1ml with carbonate buffer. This was centrifuged at 4600g for 40 mins at 4°C in a fixed head rotor. The peptide was washed once with 1ml carbonate buffer and centrifuged for a further 45 mins. The sample was collected in the adapter tube by inverting the filter and centrifuging for 5 secs. Once the gel had completely swollen, it was removed to a glass-sintered Buchaner filter and washed with 200ml 1mM HCl. Finally, the gel was washed with carbonate buffer. All preservatives should now have been removed from the gel. The gel was centrifuged for 3mins in a bench-top centrifuge and 2ml of gel slurry removed to a 5ml glass container. The washed peptide was now added to the container and allowed to interact for 1h on a spiral mixer.

After 1h, excess uncoupled peptide was washed away with 20ml carbonate buffer through a Buchaner filter. Uncoupled active groups on the gel were blocked by transferring the gel to 0.1M Tris-HCl buffer, pH 8.0. After 2h standing at room temperature, the gel was washed through a Buchaner filter with 3 alternating cycles of low and high pH buffers i.e. 3 x 5ml 0.1M acetate buffer + 0.5M NaCl, pH4 and 3 x 5ml carbonate buffer + 0.5M NaCl, pH8.3. The gel was equilibrated with PBS and stored at 4°C .

***Purification of VS1 and VS2 specific antibodies from sheep immune serum:*** The technique of fast protein liquid chromatography (FPLC) was employed to purify antibodies specific for VS1 and VS2 from sheep immune serum. Each gel coupled with VS1 or VS2 antigen was pipetted into a chromatography column and slowly

packed down by running PBS through the column at a rate of 1ml/min. Once the bed volume was level, the piston was carefully pushed down to the top of the gel excluding any air bubbles and 0.1M citrate buffer (+ 0.5M NaCl, adjusted to pH 2-5 with 1M NaOH +/- 6M urea) run through the column to gauge the background effect. The pump was returned to PBS and the sample of sheep immune serum loaded into the superloop using a syringe and adapter needle.

The sample was loaded onto the column at a flow rate of 1ml/min and fall through fractions collected in 10ml volumes. The FPLC was linked up to a spectrophotometer to allow progress of the column to be monitored. The print out was obtained at a rate of 0.2cm/ml. When the initial peak of fall through had levelled out (approximately 90-120 mins), the pump was switched to the elution buffer ( citrate buffer +/- 6M urea) and the eluate collected at the appropriate peak on the printer. The eluate was immediately neutralised by the addition of Tris.

The procedure was repeated with further additions of sheep immune serum and the fractions pooled. The samples were kept at 4°C to minimise loss of activity and dialysed over night with 3 changes of PBS using pressure dialysis through collodian filters. The samples were then aliquoted and frozen at minus 20°C.

***Quantification of affinity purified antibodies*: Total protein content:-** After dialysis of the affinity purified fractions, the samples were tested for total protein content by the Pierce Protein assay. Briefly, 10ml solution A was added to 200µl solution B to prepare the working reagent. Protein standards were prepared by diluting stock BSA solution (2mg/ml) in PBS to 8 doubling dilutions in a microtitre plate. 10µl each standard was added in duplicate to adjacent wells, whilst 10µl sample was added in triplicate to a series of adjacent wells neat and at a dilution of 1/5 in PBS. 200µl working reagent was added to each well including blank wells and the plate tapped gently to mix the contents. The plate was sealed and incubated for 30-60 mins at 37°C until a strong purple colour had developed in the positive control wells. Absorbencies were read spectrophotometrically at or near 562 nm.

**Antibody activity:-** The samples were compared to sheep immune serum in terms of concentration of VS1 and VS2 specific antibodies. The fractions eluted by citrate buffer and citrate buffer containing 6M urea were pooled for each peptide and the overall antibody activity calculated.

A 96-well flat-bottomed plate was coated with the appropriate peptide, diluted in $CO_2/HCO_3$ buffer to give 0.2µl/well, sealed and kept at 4°C overnight. The following day, the plate was washed x3 with PBS/Tween and 200µl blocking solution (10% horse serum in PBS) added to each well. The plate was sealed and the contents mixed by placing the plate on a rotary shaker for 10 mins followed by 30 mins incubation at 37°C. Meanwhile, doubling dilutions were made of the samples and of the whole sheep immune serum starting at a dilution of 1/50 to 1/6400. After washing the plate, 100µl each dilution was added to duplicate wells and the plate incubated as before.

Positive wells were detected by the addition of 100µl donkey α-sheep IgG conjugated to horseradish peroxidase SAPU), diluted 1/500 in PBS/Tween. As before, 100µl substrate solution (OPD) was added to each well to develop the coloured product . The reaction was stopped by the addition of 50µl 2.3M $H_2SO_4$ and the absorbencies read at 492 nm.

**Antibody specificity:-** The specificity of each affinity purified antibody fraction was determined by immunoblotting and by competitive ELISA:-

***Immunoblot*;** VS1 and VS2 peptides were loaded onto a 12.5% SDS gel at a concentration of 0.5µg/well. After electrophoresis at 150V for approximately 1h, the gel was transferred onto nitro-cellulose paper using the Transblot semi-dry blot apparatus. The nitro-cellulose paper was then blocked at 4°C overnight in 10% horse serum diluted in PBS.

Key: a-e, VS1 antigen

a, reagent control

b, negative control

c, positive control

d, VS1-specific antibody fraction

e, VS2-specific antibody fraction

e*-h, VS2 antigen

e*, negative control

f, positive control

g, VS1-specific antibody fraction

h, VS2-specific antibody fraction.

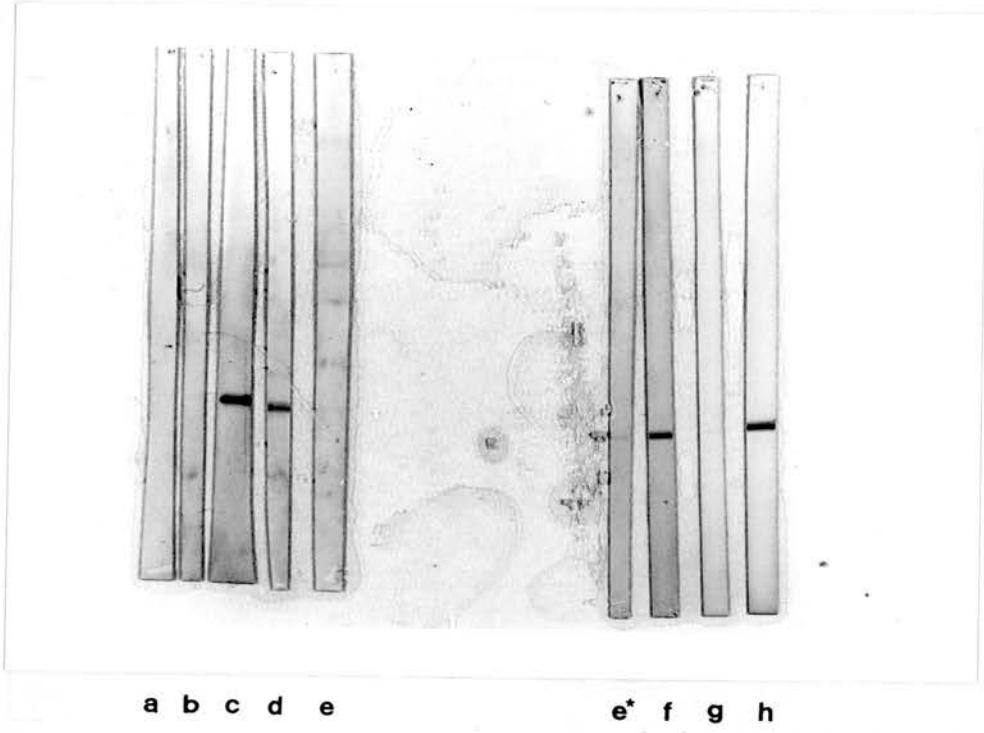



Fig 2.4. Immunoblots of affinity column purified VS fractions

Four,-5mm wide strips of nitro-cellulose paper were cut and individual strips placed in the wells of a blotting tray. Between each step of the procedure, the strips were washed 5mins x3 with PBS/Tween. Strips were probed with the affinity purified antibodies or with whole sheep immune serum at a dilution of 1/50 in 1% horse serum. The strips were agitated on a rotary shaker for 1h. After washing, 2ml donkey α-sheep/goat IgG conjugated to HRP was added to each well at a dilution of 1/1000. The strips were again agitated on a rotary shaker for 1h. The strips were then removed to a flat container and washed to remove unbound conjugate. Substrate was added to the container in the form of 10ml DAB and the reaction allowed to proceed until an intense brown band had developed at the appropriate level on the positive control strip. The reaction was terminated by washing the strips in distilled water.

***MAB 4/11 competitive ELISA*;** In order to determine if the VS2 specific fraction contained antibodies which reacted to the same epitope as the mouse monoclonal antibody 4/11, which was known to be VS2 specific, a competitive ELISA (developed by Ian Anderson) was used (Section 2.6.5.).

### 2.7.3. Peripheral blood leukocyte collection and assay

Two recombinant forms of MOMP available at the start of the project (fMOMP and mMOMP) were used to vaccinate groups of 7 sheep on 2 occasions, 3 weeks apart. Each vaccination contained approximately 50μg/ml protein and volumes of 1ml were administered subcutaneously on each occasion. Venous blood was taken from each animal approximately 21 days after second vaccination and collected into evacuated tubes containing lithium heparin (no preservative) at a final concentration of 15 units/ml. Aliquots of 1.5ml blood were mixed with 100 μl of appropriate antigen and the mixtures incubated overnight at 37°C. The following day, mixtures were centrifuged and the upper layer comprising PBLs collected.

Reactivity of PBLs was tested *in vitro* using native MOMP in two forms; semi-purified chlamydial elementary bodies and solubilised, purified MOMP. The assay

was carried out using interferon-gamma (IFN-γ) production as a measure of the reactivity of PBLs (Central Science Laboratories, Bovine γ- interferon kit). Non-specific activity of PBLs was measured by inclusion of an LPS control in the form of a B-cell mitogen (*Salmonella minnesota Re* mutant; Sigma Ltd) and a T-cell mitogen (concanavalin A).

### 2.8. Analysis of Chlamydial Infection in Mice

The mice in all experiments unless otherwise stated were infected on day 0 and killed on day 6 by exposure to $CO_2$ and cervical dislocation. The carcasses were weighed. The livers and spleens were dissected and a record kept of their weights. The organs were then crudely homogenised using scissors and divided up for different assays:

#### 2.8.1. Preparation of samples for LPS-ELISA

A small amount (0.5g) of crudely homogenised liver was added to 1ml PBS in an eppendorph. The lid was pierced and the sample boiled for 15-20 min. Once the sample had cooled, it was exposed to 4 x 5 secs bursts of sonication. The sonicator was set at 50% duty cycle, 2.5 output and fitted with a microtip. The samples are then microfuged for 15 min at 13,000 rpm before the supernatant was decanted off and stored at minus $20^0$C. The spleen and where applicable, the lungs were treated similarly, except the whole organ was used. Twice the organ weight by volume was added as PBS and the procedure continued as for the liver. Spleens and lungs only required 2 x 10 secs sonication, due to the small volumes used. Samples were diluted 1/5 or 1/10 in PBS/Tween for analysis by the LPS ELISA and 100 µl used per well.

#### 2.8.2. Culture of murine tissue

A small amount (0.5g) of crudely homogenised liver was placed in 2ml CTM and frozen at -$70^0$C until required for culture. To culture the organs, the sample was ground with a small amount of sand using a pestle and mortar. The sand was centrifuged down at low speed (100 rpm) and the supernatant decanted. The

supernatant was then diluted in warmed infection medium (RPMI + antibiotics + 2% NBCS + 1μg/ml cycloheximide) to 1/40 and 1/400 and 1ml used to infect McCoy cell monolayers in duplicate trac bottles. The tracs were centrifuged at 3000rpm for 30 min and incubated in 5% $CO_2$/ 37$^0$C for 3 days. The coverslips were fixed in methanol (10 min) and the tracs stained with 5% Giemsa (BDH Ltd) for 20 min. The coverslips were washed in tap water followed by dehydration in acetone and clearing through graded acetone/xylene mixtures. The coverslips were then mounted from xylene in a xylene based mountant (DPX) and the dark stained inclusion bodies enumerated under the light microscope.

### 2.8.3. Peritoneal exudates

***Collection:*** Peritoneal fluid was extracted from each mouse immediately after cervical dislocation to avoid posthumous changes. The mouse was pinned to a dissection board and the outer fur dampened with alcohol. A lateral incision was made through the outer skin and the skin pulled back to expose the underlying peritoneum. Using a 26 gauge needle, 2ml of 10% horse serum in PBS was injected into the peritoneal cavity. The abdomen was massaged gently and the flaps of skin at either side extended and pinned to the board to create a reservoir in which to collect the peritoneal fluid. As fluid drained to the two reservoirs, it was slowly removed using a 26 gauge needle and a 2ml syringe.

Cells from the peritoneal exudate were prepared as cytospins the same day as extraction. To do this, 200μl of peritoneal exudate was pipetted in to the funnel of the cytospin carrier and centrifuged for 5 mins. The slide was allowed to air dry (approximately 10 mins) and then fixed for one of the following procedures.

***Leishman's staining*:-** The cytospin was air dried and the lower end of the slide containing the cells covered with Leishman's stain (BDH Ltd) for 2 mins. The rest of the slide was then flooded with distilled water to give a crude 1/2 dilution of the stain. This was washed off with distilled water after 12 mins and the slides allowed to air dry completely before mounting in DPX (BDH Ltd).

***LPS-ELISA:-*** Samples of murine liver and spleen were prepared as described above (section 2.8.1) and analysed by LPS-ELISA. Peritoneal exudates were also examined by LPS-ELISA at a dilution of 1/2 in PBS/Tween.

***Immunoperoxidase staining:-*** After air drying duplicate cytospins, peritoneal cells were fixed in modified Bouin's fluid for 10 mins. The slides were then washed with tap water and thoroughly dried in air before being frozen in upright slide boxes at -20°C. Before staining by the immunoperoxidase technique described below, the cells were allowed to thaw at room temperature (approximately 10-15 mins). Sections of positive sheep placental material were used as positive controls and after mounting on microscope slides, were dewaxed in 2x 10 mins rinses with xylene. They were then rinsed in reagent grade ethanol and placed in 1% $H_2O_2$ in methanol for 30 mins to quench any endogenous peroxidase activity in the tissue. Cytospins of peritoneal exudates were also added to 1% $H_2O_2$ in methanol for 30 mins.

After 3x 5 mins washes in Tris/HCl, the slides were placed in 10% egg albumin for 30 mins. They were then rinsed in distilled water and washed as before. The slides were carefully wiped around the tissue sections to remove excess fluid and incubated with primary antibody overnight at 4°C in a humidity chamber. Primary antibody comprised purified sheep IgG from an immune animal post-abortion and was used at an optimal working dilution of 1/160 in high salt buffer (7µl primary antibody in 1.113ml high salt buffer).

The following day, the slides were washed 3x 5 mins with Tris/HCl buffer and blotted to remove excess solution. The slides were then incubated for 90 mins in a humidity chamber with donkey α-sheep/goat IgG conjugated to HRP (5µl conjugate diluted in 1ml of high salt buffer). Titration of the conjugate indicated an optimal working dilution of 1/200. The slides were washed 3x 5mins with Tris/HCl and finally incubated with peroxidase substrate solution- (diaminobenzidine (DAB); 1 tablet dissolved with 1 tablet of $H_2O_2$ in 5ml of distilled water; Sigma Chemicals

Ltd.) until brown staining was just visible (approximately 5-8 mins). Excess DAB was drained into a container and the sections were washed in tap water to stop any further colour development. The slides were counter-stained with haematoxylin and Scott's tap water, dehydrated in graded alcohols and cleared in xylene. The slides were finally mounted from xylene in DPX (BDH Ltd) and viewed under the light microscope.

# CHAPTER 3

## INDUCTION AND ANALYSIS OF CHLAMYDIAL INFECTION IN MICE

## 3.1. Introduction

Animal models have been used extensively to study the immunopathology of human chlamydial diseases (Mount *et al.,* 1973; Rank *et al.,*1979; Rank and Barron, 1983; Tuffrey *et al.,* 1986a; Patton *et al.,* 1989; Kaukoranta-Tolvanen *et al.,* 1993; Westbay *et al.,* 1994). Ovine abortifacient *C. psittaci* infection has also been studied in mouse models using both pregnant and virgin mice to: investigate the kinetics of infection (Lammert, 1982); to compare the virulence of different strains of *C. psittaci* (Buzoni-Gatel and Rodolakis, 1983; Anderson, 1986; Rodolakis and Lantier, 1989); to determine susceptibilities of various inbred strains of mice (Buzoni-Gatel *et al.,*1994); and to assess the contribution of cytokines to resolution of infection (McCafferty *et al.,* 1994). The first experiments in mice to assess the efficacies of chlamydial vaccines were performed as early as 1954 (McEwan and Foggie), using intranasal inoculation and analysis of lung consolidation as a measure of chlamydial infection. Mouse protection models were not used extensively thereafter, until twenty years later when the efficacy of the original vaccine was rapidly declining. The potency of a live vaccine containing a temperature-sensitive mutant strain of *C. psittaci* (abortifacient, AB7) is being investigated in mice (Rodolakis, 1983) and represents one approach to this problem. This chapter describes the development of two mouse models for investigation of potential vaccines, using intraperitoneal inoculation to infect susceptible mice.

An integral part of any mouse model, is the accurate measurement of *in vivo* infection levels. Chlamydial infection was originally determined by visual assessment of infected organs or subsequently by titration of chlamydial infectious bodies using either the plaque assay method (Banks, 1970) or the Trac method. In both the latter cases, only chlamydiae in the infectious elementary body stage of development are detected. The techniques require between 3 days (Trac method) and 2 weeks (Plaque assay) to complete and final quantification involves identification of individual inclusions under the microscope. More recently a "sandwich" ELISA has been developed (G. E. Jones *et al.,* unpublished; Graham *et al.,* 1995) which detects

chlamydial lipopolysaccharide (LPS) and allows rapid and accurate quantification of chlamydiae. The technique was originally developed for detecting *C. psittaci* in sheep placentas. However in this chapter, adaptations of the tissue processing stages are described which allow its use with mouse liver, spleen and lung.

## 3.2. Materials and Methods.

### 3.2.1. Quantifying chlamydial infection in mice.

#### 3.2.1.1. ELISA procedure.

The "sandwich" LPS-ELISA technique was utilised in which a mixture of two genus-specific, anti-LPS monoclonal antibodies, 13/4 and 13/5 (1:1 ratio) was used in the capture phase and 13/4 conjugated to horseradish peroxidase in the detection phase. The epitope against which the MAbs react is repeated along the O-chain of the LPS molecule, which allowed the use of the same MAbs in both the capture and detection phases.

M129B ELISA plates (Greiner Labortechnik Ltd, Dursley, UK) were coated with 100µl/well of MAbs 13/4 and 13/5 (1:1 ratio) to give a final dilution of 1/2500 in carbonate buffer, pH 9.6. The plates were stored at 4°C overnight and the following day the coating MAbs were expelled from the wells and the plate washed with three changes of PBS containing 0.05% Tween 20 (Sigma). After blotting the plate dry, each well was blocked with 200µl of 10% Marvel, diluted in carbonate buffer. The plate was covered and incubated at 37°C for 1h after which time the wells were washed as before. Samples were diluted in PBS-Tween (0.05%) to give a working dilution of 1/5. Each sample was added to duplicate wells in 100µl volumes and the plate incubated as before. *Salmonella minnesota* Re 595 LPS (Sigma) was used as the positive control. After 1h at 37°C, the plate was washed and 100µl of MAb 13/4 conjugated to horseradish peroxidase was added to each well diluted to 1/160 (or alternative for other conjugate batches) in PBS-Tween (0.05%). After a final incubation at 37°C for 1h, the plate was washed and 100µl/well of orthophenylenediamine dihydrochloride (OPD, 0.8mg/ml;Sigma) in citrate phosphate buffer, pH5.0, containing 0.8µl/ml of 30% $H_2O_2$ added to each well. After approximately 10min incubation in the dark, the colour reaction was stopped by the addition of 50µl 2.3M $H_2SO_4$ to each well. Absorbances were read at 492-nm using

a Titertek Multiscan ELISA reader (Flow, High Wycombe, UK) and converted to units of concentration { ng of LPS/g of wet tissue }.

### 3.2.1.2. Adaptation of the LPS-ELISA technique to detect *C. psittaci* in mouse tissue.

Various processing techniques were tested in order to obtain the maximum levels of chlamydial LPS from mouse tissue. Extraction of chlamydial LPS from infected liver tissue was examined by both detergent and physical methods, which included solubilisation with either:-

1) Tween 20 (Sigma); 0.05-0.5%,

and/or 2) sodium deoxycholate; 0.1-1%

and disruption of intact cells by sonication and/or boiling.

#### 3.2.1.2.1. Procedure

Briefly, a pool of infected mouse liver was crudely homogenised with sterile scissors and 0.5g amounts weighed into 1.5ml eppendorf tubes. The contents of each eppendorf were mixed with 1ml of solution as shown in Table 3.1. and then processed by one of three methods:-

1) boiled for 10 mins,
2) sonicated for 2x10sec., (50% duty cycle, 2.5 output) or
3) boiled for 10 min. followed by sonication as in 2.

After each treatment, the samples were microcentrifuged for 5min. and the supernatants decanted for analysis by LPS-ELISA. Non-infected mouse tissue was also processed to identify treatments which gave high background results in the ELISA. Net absorbances after subtraction of negative control values (non-infected

tissue) are given in Table 3.1. Optical density values were not converted to units of concentration at this stage.

### 3.2.1.3. Correlation between *in vitro* culture and LPS-ELISA of infected mouse tissue.

The LPS-ELISA was considered as an alternative method to culturing samples *in vivo,* in order to quantify chlamydial infection in internal organs. A comparison was made between the 2 methods using *C. psittaci* strain (avian Z10).

#### 3.2.1.3.1. Procedure for culturing samples.

*In vivo* culturing was performed in McCoy cell monolayers, which had been seeded at $2x10^5$ cells/ml and allowed to monolayer overnight in Tracs. Each tissue sample (0.5g) had been stored in 4ml CTM at -70°C. Samples were thawed and homogenised with a small quantity of sterile sand using a pestle and mortar, until a uniform consistency was obtained. The sand was gently sedimented by centrifuging at 100rpm for 5min., after which the supernatant was decanted into a sterile container. A 1/400 dilution of the supernate was made in RPMI medium (pre-warmed to 37°C) containing 5% serum and 1μg/ml of cylcoheximide. Duplicate Tracs were infected with 1ml of diluted sample and centrifuged for 30min. at 3000rpm. Tracs were then incubated at 37°C for 3 days before fixing in methanol and staining in 5% Giemsa solution. Inclusions were counted per coverslip and the mean number for duplicates was calculated. Correlations are shown in Fig. 3.2. comparing infection titre of samples with concentration of chlamydial LPS.

## 3.3. Results

Treatment of liver samples by boiling and sonicating resulted in consistently high values in the LPS-ELISA (mean 1.120; sem 0.166) regardless of the solute (Table 3.1.). Sonication alone resulted in high background contamination which reduced the

net values to zero, except where LPS levels were greater than the background value (0.1% D.O.). This would not therefore be a suitable method for detecting low levels of LPS. Boiling alone did not result in high background, however the overall LPS levels were lower than in equivalent samples which were both boiled and sonicated ($p<0.01$). One exception to this was 0.5% D.O., which resulted in a high LPS level with boiling alone. No other concentrations of D.O. or Tween gave comparable results with this method, whilst boiling followed by sonication gave generally higher values ($p<0.01$) in both cases.

The highest LPS levels were detected by boiling and sonicating samples with PBS compared to PBS + Tween or D.O. ($p<0.01$).

***Table 3.1.*** *Extraction and detection of chlamydial LPS from infected mouse livers by LPS ELISA.*

| **Treatment ;** | **Absorbance at 492nm** | | |
|---|---|---|---|
| **(PBS +)** | **boil** | **sonicate** | **boil/sonicate** |
| **PBS only** | O.061 | 0.041 | 1.787 |
| **0.05% Tween** | 0.097 | 0.000 | 1.664 |
| **0.1% Tween** | 0.174 | 0.000 | 1.236 |
| **0.2% Tween** | 0.194 | 0.000 | 1.174 |
| **0.5% Tween** | 0.643 | 0.000 | 1.122 |
| **mean background** | 0.047 | 2.316 | 0.006 |
| **0.1% D.O.** | 0.074 | 1.748[a] | 1.498 |
| **0.5% D.O.** | 1.714 | 0.000 | 0.703 |
| **1.0% D.O.** | 0.664 | 0.030 | 0.375 |
| **0.05% Tween +1% D.O.** | 0.022 | 0.000 | 0.524 |
| **mean background** | 0.000 | 0.490 | 0.060 |

**PBS** - *Phosphate buffered saline;* **Tween** - *Tween 20 (Sigma);* **D.O.** - *sodium deoxycholate.*

- *Samples tested in duplicate*
- *Mean background absorbance from uninfected samples subtracted*
- *samples mixed with solution (0.5g crudely homogenised tissue per ml of solution).*
- *samples boiled (10min), or sonicated (2x10sec; 50% duty cycle,2.5 output), or boiled (10min), and sonicated, followed by microcentrifugation (5min, 13000 rpm*
- [a] *high background (0.490nm)*

***Statistical Analysis (Student's t-test):-***

*Between physical treatments; Boil/sonicate> boil ($p<0.01$)*

*Between chemical treatments; Tween> D.O. ($p>0.05$), PBS> Tween; ($p<0.01$).*

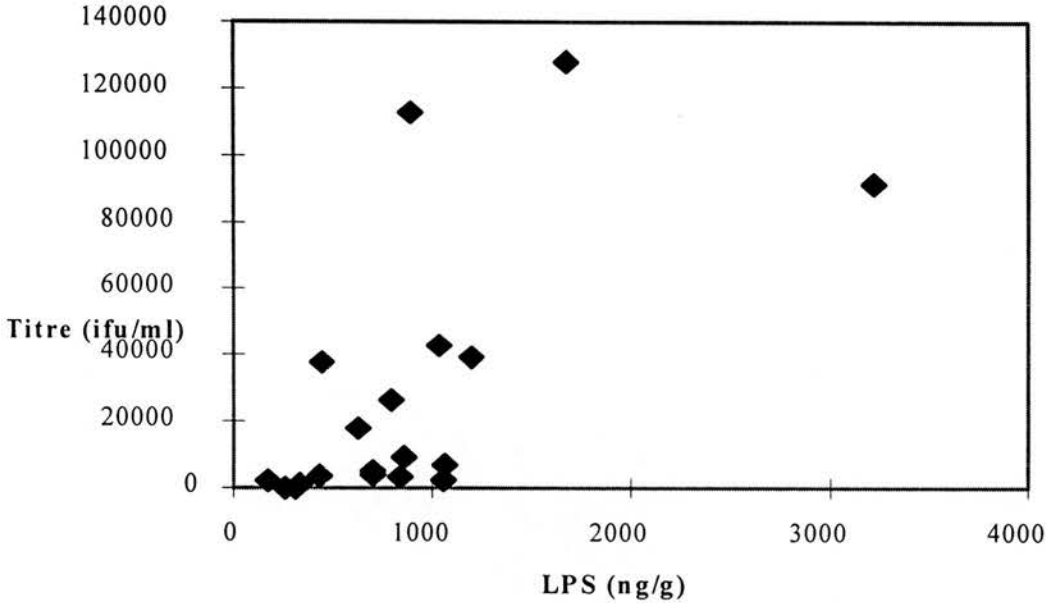



**Fig. 3.1.** Correlation between 2 methods of estimating organ infection with *Chlamydia* spp. :- a) *in vitro* culture ; b) LPS-ELISA.

- *X axis = concentration of chlamydial LPS in mouse liver (ng/g). Y axis = number of infectious chlamydiae (ifu/ml).*
- *Correlation coefficient = 0.64; significant correlation between culture and LPS-ELISA results ($p<0.01$).*

No advantage was gained by adding Tween to PBS and there was a trend towards a negative correlation between LPS levels and concentration of Tween in the solution (correlation coefficient , -0.68). A similar effect also occurred with the addition of increasing concentrations of D.O. to PBS (correlation coefficient, -0.96). However, neither statistic was significant ($p>0.05$) since sample sizes were small.

In summary, the optimal processing technique for mouse tissue was boiling for 10 min. in PBS (0.5g tissue/ml PBS), followed by 2x10sec sonications using a tapered microtip and collecting the supernatant after 5min. microcentrifuging at 13000rpm. All subsequent mouse samples were treated this way for analysis by LPS-ELISA.

### 3.4. Induction of a systemic infection in ADRA/CBA mice

#### 3.4.1. Model 1- Active Immunisation.

Active immunisation of mice was considered to assess the efficacies of recombinant vaccines. Experiments were designed using chlamydial inocula and ADRA/CBA mice. Initial parameters to be standardised were, titre of inoculum, day of killing and the route of infection.

##### 3.4.1.1. Experimental proccdurc

*1) Infection of mice*

Forty eight, 12 week old ADRA /CBA mice were randomly allocated to 4 groups of 20 and 1 group of 8 mice with a restriction on age and sex. The mean weight of each group was recorded and showed no significant differences between groups. The mice were bled from the right lateral tail vein and the sera assayed by indirect ELISA (Materials and Methods, section 2.6.1.) to ensure negativity for anti-chlamydial antibodies. After allowing the mice to become accustomed to their new surroundings (2-3 days), each was inoculated intraperitoneally with 1ml of tissue-grown

chlamydial inoculum (Section 2.3.), such that group 1 received $1x10^{6}$ ifu and groups 2, 3 and 4 received $1x10^{5}$ ifu, $1x10^{4}$ ifu, and $1x10^{3}$ ifu, respectively; group 5 received no inoculation. Four mice from each group were killed daily between days 4 and 8 post-inoculation (inclusive), except group 5 in which 4 mice were killed only on days 4 and 8 (Table 3.2.).

### 3.4.1.2. Results

#### 3.4.1.2.1. LPS levels in internal organs

The extent of infection in the liver, lung and spleen was estimated using the LPS-ELISA to detect chlamydial LPS in these organs. Infection was only detected in Group 1 ($1 \times 10^{6}$ ifu/ml) using this technique, therefore only data for this group and that of the negative control group (Group 5) are shown in Fig. 3.3. Inoculum injected at a titre of $1 \times 10^{5}$ ifu/ml or less, did not result in levels of infection which were detectable by this assay .

***Table 3.2.*** *Experimental design defining the parameters of infection in Model 1.*

| group | challenge titre (ifu/ml) | n | number of mice killed on day | | | | |
|---|---|---|---|---|---|---|---|
| | | | day 4 | day 5 | day 6 | day 7 | day 8 |
| 1 | $1 \times 10^6$ | 20 | 4 | 4 | 4 | 4 | 4 |
| 2 | $1x\ 10^5$ | 20 | 4 | 4 | 4 | 4 | 4 |
| 3 | $1 \times 10^4$ | 20 | 4 | 4 | 4 | 4 | 4 |
| 4 | $1 \times 10^3$ | 20 | 4 | 4 | 4 | 4 | 4 |
| 5 | none | 8 | 4 | 0 | 0 | 0 | 4 |

***n*** *number of mice per group at start of experiment (day 0).*

- *ADRA/CBA mice; 6-16 weeks old, 18-25g weight.*
- *each group received 1ml of chlamydial inoculum (Batch A) at stated titre by intraperitoneal injection on day 0.*
- *4 mice from each group were killed daily between days 4 and 8 post-infection (p.i.), except Group 5 in which 4 mice were killed only on days 4 and 8.*
- *organ infection analysed by LPS-ELISA.*

Levels of chlamydial infection in Group 1 varied over the 5 days of sampling. Significant levels of infection were detected in the livers ($p<0.01$) and spleens ($p<0.05$) of group 1 by day 4 p.i., but not in the lungs ($p<0.01$) until day 5. As the mean level of infection increased in the liver (day 7 p.i.), the results became statistically non-significant ($p>0.05$) due to substantial between sample variations. Likewise, infection levels in the spleen were statistically non-significant on day 7 and only marginally significant ($p<0.05$) on day 5. Infection of the lung was, however, significant ($p<0.05$) at all samplings post-infection except on day 4, when chlamydial levels were low. No significant differences were calculated between infection levels in the three organs, except on day 4 when infection in the lung was significantly less than in the liver ($p<0.01$) or spleen ($p<0.05$). The optimum time to detect significant ($p<0.05$) levels of chlamydiae in internal organs was therefore chosen as day 6, since significantly ($p<0.01$) high infection levels on this day were accompanied by low validity in all three organs.

#### *3.4.1.2.2. Spleen weights*

Although LPS was not detected in Groups 2 to 4, spleen weights of mice in these groups and in Group 1, were significantly higher on days 4 ($p<0.05$) and 8 ($p<0.01$) than spleen weights in uninfected controls (Table 3.3.). There were no significant differences ($p>0.05$) between spleen weights of groups 1 to 4 on day 4. However, on day 8, group 2 spleens were significantly ($p<0.01$) heavier than those in group 1. This was interesting since group 1 had the highest levels of splenic infection (Fig.3.3.). Spleen weights on day 8 were significantly higher in groups 1 ($p<0.05$) and 2 ($p<0.001$) compared with weights on day 4.

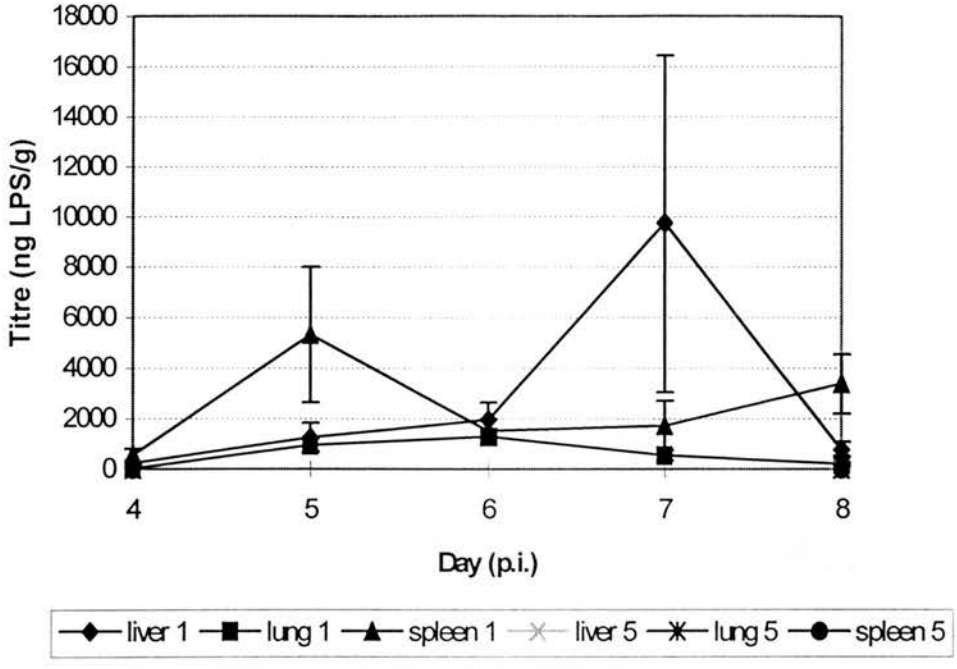



***Fig 3.3.*** *Chlamydial LPS levels in internal organs in Model 1.*

- *n = 4.*
- *Group 5 = uninfected controls (all values zero)*
- *mice in Group 1 inoculated intraperitoneally with $1x10^6$ ifu/ml viable chlamydiae on day 0.*
- *chlamydial infection estimated by LPS-ELISA (ng/g).*

***Statistical Analysis (Student's t-test):-***

- *LPS levels significantly higher ($p<0.05$) than in controls (group 5) on Day 4 (spleen) and on Day 5 (liver + spleen).*
- *LPS levels significantly higher ($p<0.01$) than in controls (group 5) on Day 4 (liver), Day 5 and 7 (lung), Day 6 and 8 (liver, lung + spleen).*

### 3.4.1.2.3. Clinical signs

A set of criteria were taken to standardise the clinical signs associated with increasing degrees of chlamydial infection. These were as follows, in order of increasing severity:-

Clinical signs associated with infection of mice with viable chlamydiae

*ruffled fur*
*hunched posture*
*reluctance to move*
*white/closed eyes*
*comatose/moribund*
*dead*

Each mouse which displayed abnormal signs post-infection, was classified under one of the designated headings (Table 3.4.).

Mice rapidly displayed signs of illness soon after injection, with damp looking, ruffled fur over the first 2 days post-infection. By day 5 post-infection, one mouse in group 1 had developed a white eye, a classical sign of chlamydial infection in mice. Day 6 post-infection, was marked by all 12 mice in group 1 showing ruffled fur with additionally, 1 mouse which demonstrated a hunched posture and another which was reluctant to move. On day 7 post-infection, the mouse which had developed a white eye died. No other deaths occurred. Group 2 ($1 \times 10^5$ ifu/ml), was the only other group to show any signs of infection, with 1 mouse having ruffled fur on days 3 and 4.

***Table 3.3.*** *Mean spleen weights of mice on days 4 and 8 after infection with abortifacient* C. psittaci, *strain S26/3 in Model 1.*

| group | challenge | n | mean spleen weight (mg) | | | |
|---|---|---|---|---|---|---|
| | | | day 4 | | day 8 | |
| | titre (ifu/ml) | | mean[a] | *sem* | mean[b] | *sem* |
| 1 | $1x10^6$ | 4 | 93[c] | *11* | 165[c] | *26* |
| 2 | $1x10^5$ | 4 | 115[d] | *16* | 300[d] | *23* |
| 3 | $1x10^4$ | 4 | 160 | *15* | 203 | *37* |
| 4 | $1x10^3$ | 4 | 143 | *28* | 203 | *17* |
| 5 | none | 4 | 46 | *8* | 75 | *12* |

***n*** *number of samples contributing to mean value.*

- *mice infected intraperitoneally on day 0 with 1ml challenge at stated titre. Four mice per group killed on days 4 and 8 post-infection and spleens weighed as wet tissue.*

***Statistical Analysis (Student's t-test):-***

[a] *spleen weights in groups 1-4 significantly greater ($p<0.05$) than in group 5 (control). No significant difference ($p>0.05$) between spleen weights in groups 1-4.*

[b] *spleen weights in groups 1-4 significantly higher ($p<0.05$) than in group 5 (control). Spleen weights in group 1 significantly ($p<0.01$) lower than in group 2. No significant differences ($p>0.05$) between spleen weights in groups 1, 3 or 4.*

[c] *spleen weights on day 4 significantly ($p<0.05$) lower than on day 8.*

[d] *spleen weights on day 4 significantly ($p<0.001$) lower than on day 8.*

***Table 3.4.*** *Numbers of mice in Group 1 showing specific clinical signs of illness post-inoculation with viable chlamydiae.*

| Symptom | Number of mice | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *Day 0* | *Day 1* | *Day 2* | *Day 3* | *Day 4* | *Day 5* | *Day 6* | *Day 7* | *Day 8* |
| normal | 20 | 15 | 15 | 13 | 13 | 9 | | | |
| ruffled fur | | 5 | 5 | 7 | 7 | 6 | 12 | 7 | 4 |
| hunching | | | | | | | 1 | | |
| reluctance to move | | | | | | | 1 | | |
| white/closed eye | | | | | | 1 | | | |
| comatosed/ moribund | | | | | | | | | |
| dead | | | | | | | | 1 | |
| Total number of mice | 20 | 20 | 20 | 20 | 20 | 16 | 12 | 8 | 4 |

- *mice injected with 1ml of $1x10^6$ ifu/ml, chlamydial inoculum (day 0).*

- *clinical assessments made up to 8 days post-infection and recorded under one of the above headings (increasing order of severity).*

### 3.4.1.3. Route of inoculation

The route of inoculation has been investigated previously with different strains of *C. psittaci* (Buzoni-Gatel and Rodolakis , 1983). An abortifacient strain was shown to be virulent when injected either intraperitoneally or via the footpad. However, inoculation via the footpad resulted in a slight inflammatory reaction which was associated with reduced infectivity of the less virulent intestinal strains of *Chlamydia* spp. The strain used in this study, S26/3 has been categorised as invasive although not as highly as other strains (Rodolakis *et al.*, 1989). In order to induce maximal organ infection therefore, intravenous challenge using approximately $1x10^6$ - $1x10^7$ ifu/0.1ml was compared to the previous intraperitoneal challenge (Fig. 3.3.).

***Table 3.5.*** *Experimental design defining the parameters of intravenous infection.*

| group | challenge titre (ifu/0.1ml) | n | number of mice killed on day | | | |
|---|---|---|---|---|---|---|
| | | | day 4 | day 5 | day 6 | day 7 |
| 1 | $1 \times 10^6$ | 26 | 6 | 6 | 7 | 7 |
| 2 | $1 \times 10^7$ | 23 | 5 | 6 | 6 | 6 |

***n***, *number of mice per group at start of experiment (day 0).*

- *each group received 0.1ml of chlamydial inoculum at $1x10^6$ or $1x10^7$ ifu/ml by intravenous injection on day 0.*
- *5-7 mice from each group were killed daily between days 4 and 7 post-infection (p.i.).*

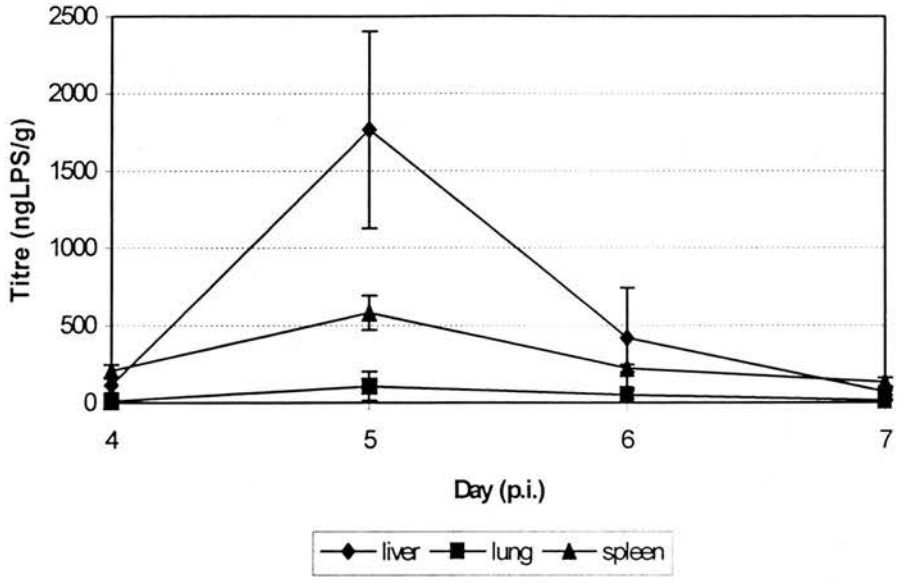



*Fig 3.4. Chlamydial LPS levels in internal organs after intravenous inoculation.*

- ***n**, number of samples contributing to mean value.*
- *mice infected intravenously with $1x10^7$ ifu/0.1ml.*
- *analysis of organ infection by LPS-ELISA (ng LPS/g wet tissue).*
- *no LPS values greater than 83 ng/g (sem 26) detected in group 1.*

***Statistical Analysis (Student's t-test):-***

*LPS levels significantly higher ($p<0.05$) than control (group 5, Fig. 3.3.) on Day 4 in the liver and lung.*

*LPS levels significantly higher ($p<0.01$) than control (group 5, Fig. 3.3.) in spleen on Days 4, 5, 6 and 7; lung on Day 7 and liver on Days 5 and 7.*

[c] *LPS levels not significantly different ($p>0.05$) to control (group 5, Fig. 3.3.).*

The results of infecting CBA/ADRA mice intravenously can be seen in Table 3.5. Significant chlamydial LPS was detected in the liver ($p<0.05$) and spleen ($p<0.01$) by day 4 post-infection as in the previous experiment. In contrast to inoculation via the peritoneal route however, peak LPS levels occurred on day 5 in the spleen and liver ($p<0.01$). Although LPS levels in the liver on this day were higher than those in the spleen, the difference was not statistically significant ($p>0.05$) . Overall, intraperitoneal inoculation was the preferred route of administering chlamydial challenge, since it was technically a more simple procedure and resulted in significant ($p<0.01$) LPS levels in the spleen, lungs and liver detectable by the LPS-ELISA.

### 3.4.2. Model 2 - Passive protection and infection.

A second model was assessed in order to compare its action in protection studies against Model 1. In this case, *C. psittaci* was incubated with an equal volume of non-immune sheep serum for 45 min at 37°C, before injecting 1ml of the reaction mixture into mice intraperitoneally. Because of the delay between thawing and injecting the inoculum, the use of a higher titre of chlamydiae was examined to compensate for those dying during the 45 min incubation period.

#### 3.4.2.1. Experimental procedures.

##### 3.4.2.1.1. Comparison of inoculum titres.

Two separate experiments were compared in which the same batch of inoculum was used, but at different titres. Both experiments involved incubating the inoculum with an equal volume of heat inactivated non-immune sheep serum at 37°C for 45 min.

### 3.4.2.2. Results.

Incubating the inoculum with non-immune sheep serum for 45 min. at 37°C, significantly ($p<0.01$) reduced LPS levels in the liver compared to immediate injection as in Model 1 (Fig. 3.5). LPS levels were significantly enhanced ($p<0.01$) by increasing the inoculum titre to $2x10^6$ ifu/ml. The higher titred inoculum did not result in significantly different LPS levels ($p>0.05$) to those seen in Model 1. In contrast, splenic LPS levels occurred at significantly ($p<0.01$) lower levels compared to liver LPS levels when mice were inoculated with $2x10^6$ ifu/ml chlamydiae. However, the weaker inoculum did not result in any difference between organ LPS levels ($p>0.05$).

## 3.5. Variation in infection levels between experiments

### 3.5.1. Model 1.

In order to assess the repeatability of Model 1, data was collated from 7 experiments performed on separate occasions. Infection levels in the liver and spleen of mice injected with $1x10^6$ ifu/ml intraperitoneally, were compared between experiments, between inoculum batches and, where mice had previously been immunised with placebo vaccine - between adjuvants.

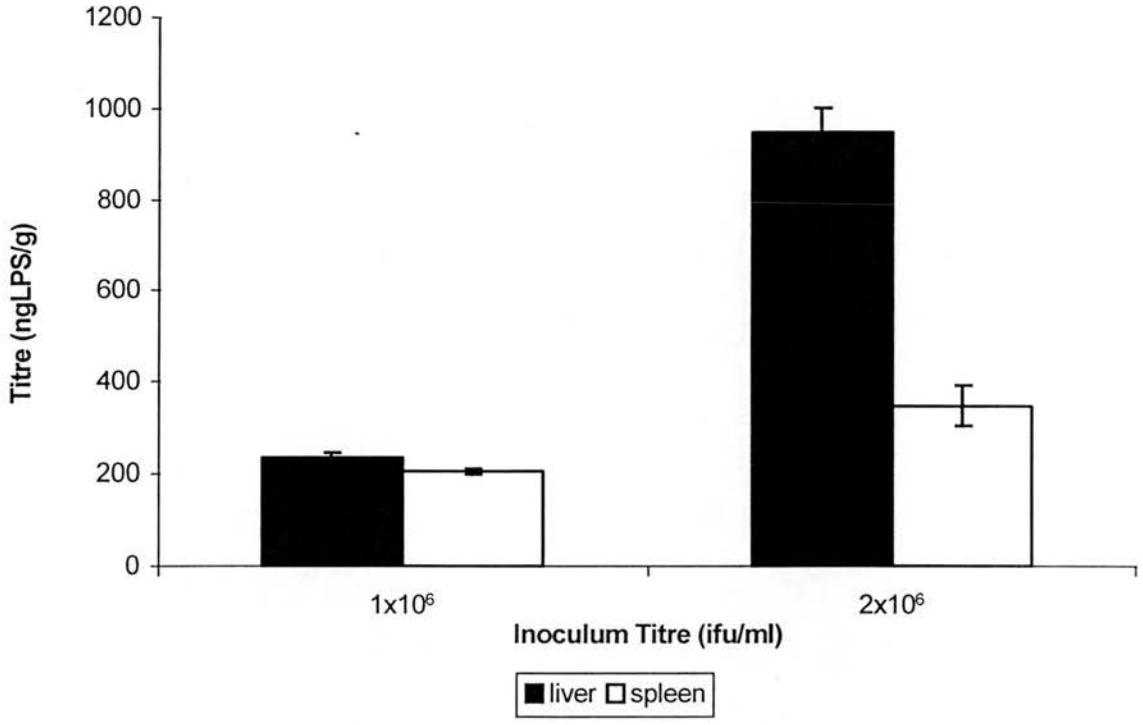



**Fig. 3.5** *Comparison of Inoculum Titres in Model 2*

- ***n** = 12, inoculum titre 1x10⁶; **n** = 9, inoculum titre 2x10⁶*
- *inoculum mixed with sheep non-immune serum (1:1) ratio; incubated at 37°C for 45 min.*
- *1ml injected intraperitoneally into mice.*
- *Mice killed on day 6 post-injection; organs analysed by LPS-ELISA.*
- *inoculum batch **C** - 11th passage.*

***Statistical Analysis (Student's t-test):-***

Liver LPS levels significantly higher with $2x10^6$ ifu/ml inoculum compared to $1x10^6$ ifu/ml ($p<0.01$).

### 3.5.1.1. Results - Model 1.

Comparing LPS levels in spleen and liver between the 7 experiments (Table 3.6.), it was found that consistency was low and in particular, there was a significant difference ($p<0.01$) in liver infection between the experiments. LPS levels in the spleen were more consistent (no significant difference; $p>0.05$). especially when analysis was restricted to experiments using a single inoculum (Batch A). Variability in liver LPS levels however, remained high ($p<0.01$). Statistical analysis revealed no correlation between LPS levels in the spleen and liver when mice were injected intraperitoneally with $1x10^6$ ifu/ml of chlamydial inoculum (correlation coefficient, 0.33; no significant correlation, $p>0.05$). Consistency in spleen LPS levels between experiments could be extended to include experiments performed with any of inocula A, B or C, but did not include inoculum D which gave significantly ($p<0.01$) lower levels of LPS. The six experiments showing no significant differences in spleen LPS levels (1, 3a, 3b, 5, 9 and 11) contained a further variable, namely alternative adjuvants where challenge with chlamydiae had been pre-empted by 2 placebo vaccinations. Therefore, the results also indicated that there was no significant ($p>0.05$) effect on spleen LPS levels of immunising mice with placebo vaccine in a variety of adjuvants, compared to each other and to Experiment 1 (no immunisations pre-challenge).

To summarise, infection of mice intraperitoneally with $1x10^6$ ifu/ml produced levels of LPS and presumably, of infection in the spleen which could be reproduced in subsequent experiments using the same inoculum batch. This was not the case for the liver. In all, three out of four inoculum batches produced comparable results in the spleen, whilst one batch failed to induce sufficient levels of LPS and was significantly ($p<0.01$) lower than the others. No significant ($p>0.05$) effect was produced by immunising mice with placebo in a variety of adjuvants, (Marcol Arlacel A + Alhydrogel, Montanide ISA 50 or Montanide ISA 50 + Alhydrogel) prior to challenge, compared to no immunisation. This indicated a lack of either protection or enhancement of infection due to adjuvant alone.

### 3.5.2. Model 2

Variation in organ LPS levels between experiments, was estimated using nine separate experiments performed by the procedure for Model 2. Briefly, 8-15 week old mice were infected intraperitoneally with $2x10^6$ ifu/ml chlamydiae, containing 25% non-immune sheep serum. In addition, Experiments 8 and 19 contained guinea pig serum at 5% (final volume). The reaction mixtures (chlamydiae + 25% serum diluted in RPMI) were incubated at 37°C for 45 min. prior to injection. Data from the 9 experiments were compared and the variation between experiments calculated in terms of organ LPS values. The liver and spleen were treated separately. Variation arising from the use of different inoculum batches was assessed, as was variation between infection levels in experiments using the same inoculum. Correlation coefficients were calculated to determine whether any correlation existed between LPS levels in the liver and spleen.

#### 3.5.2.1. Results - Model 2.

The coefficient of correlation (0.49; $p>0.05$) indicated that there was no relationship between LPS levels in the liver and those in the spleen of individual animals infected by the method used in Model 2 (Table 3.7). In order to assess if infection occurred randomly in one or both organs, 5 experiments were compared in which the same inoculum (batch C) had been used. There was a significant ($p<0.001$) degree of variation in spleen LPS levels between these 5 experiments, however levels of LPS in the liver were more consistent and variation between experiments was not significant ($p>0.05$). A wider comparison was performed to calculate the variation in organ LPS values over all 9 experiments. A total of 5 different inoculum batches (A, B, C, D and F) were used in this range of experiments. The results showed significant variation in LPS levels of both the liver ($p<0.05$) and spleen ($p<0.001$), between experiments using different inoculum batches.

*Table 3.6. Variation in organ LPS levels in Model 1.*[1]

| [1]Expt. | adjuvant | inoculum | *Chlamydial LPS (ng/g)* spleen | | | liver | | |
|---|---|---|---|---|---|---|---|---|
| | | | mean | *sem* | *n* | mean | *sem* | n |
| 1 | *none* | *A* | 1536 | *473* | 3 | 1984 | *663* | 3 |
| 3a | *M/A + Al.* | *A* | 1288 | *726* | 3 | 6467 | *4042* | 3 |
| 3b | *Mont. + Al.* | *A* | 6091 | *4074* | 6 | 16864 | *5717* | 6 |
| 5 | *Mont.* | *A* | 1081 | *408* | 10 | 232 | *52* | 10 |
| 9 | *Mont. + Al.* | *B* | 1245 | *155* | 9 | 770 | *99* | 10 |
| 11 | *Mont. + Al.* | *C* | 1948 | *495* | 10 | 847 | *162* | 10 |
| 6 | *Mont. + Al.* | *D* | 275 | *67* | 20 | 12 | *2* | 20 |

- ***n**, number of samples contributing to mean value*
- *mice received 2 placebo vaccines, 3 weeks apart, 3 weeks prior to inoculation with live chlamydiae at $1x10^6$ ifu/ml (except expt. 1).* ***Placebo vaccine adjuvant**:- **M/A**, Marcol Arlacel/A; Mont, Montanide ISA 50; **Al**, Alhydrogel.*
- ***Inocula**:- **A** - $7^{th}$ passage; **B** - $8^{th}$ passage; **C** - $11^{th}$ passage; **D** - $8^{th}$ passage + $10^{th}$ passage (1:1 ratio).*

***Statistical Analysis (Student's t-test):- variation between experiments;** spleen, $p>0.05$; liver, $p<0.01$; **variation within inoculum batch (A);** spleen, $p>0.05$; liver, $p<0.01$. **variation between inoculum batches (A, B, C);** spleen, $p>0.05$; liver, $p,0.01$; **variation between adjuvants;** spleen, $p>0.05$; liver $<0.01$*

In summary, statistical analysis of LPS values in 9 experiments using Model 2, demonstrated that infection in the liver could be achieved at a constant level in different experiments, but that this was restricted to the use of one batch of inoculum for all such experiments. Consistency was lost when a different batch of inoculum was used, even when the same titre of chlamydiae (quantified in tissue culture) was present. LPS levels in the spleen, in contrast to the liver, were highly variable between experiments using this method.

**Conclusions**

Models 1 and 2 both displayed consistencies in levels of organ infection, after intraperitoneal inoculation of mice with $1x10^6$ or $2x10^6$ ifu/ml, respectively. However, whereas in Model 1, the levels of chlamydial LPS in the spleen remained constant between experiments, in Model 2 consistent LPS levels were only seen in the liver.

***Table 3.7.*** *Variation in organ LPS levels in Model 2.*[1]

| [2]Expt. | Inoculum batch | *Chlamydial LPS (ng/g)* | | | | | |
|---|---|---|---|---|---|---|---|
| | | spleen[a] | | | liver[b] | | |
| | | **mean** | ***sem*** | ***n*** | **mean** | ***sem*** | **n** |
| 8 | *A* | **840** | *152* | 8 | **3259** | *1423* | 8 |
| 7 | *D* | **82** | *97* | 10 | **378** | *134* | 10 |
| 10 | *B* | **249** | *79* | 7 | **367** | *134* | 7 |
| 12 | *C*[c] | **1965** | *246* | 9 | **2286** | *409* | 10 |
| 15 | *C*[c] | **ND.** | ND. | ND. | **3034** | *1235* | 10 |
| 18 | *C*[c] | **950** | *52* | 9 | **347** | *44* | 9 |
| 19 | *F* | **1703** | *275* | 5 | **2280** | *497* | 5 |
| 20 | *C*[c] | **648** | *128* | 10 | **1256** | *162* | 10 |
| 22 | *C*[c] | **1919** | *720* | 4 | **1000** | *241* | 4 |

- ***n****, number of samples contributing to mean value*
- *mice inoculated intraperitoneally with 1ml of live chlamydiae at* $2x10^6$ *ifu/ml, pre-incubated with non-immune sheep serum (1:1 ratio).*
- *Inocula:-* ***A*** *-* $7^{th}$ *passage;* ***B*** *-* $8^{th}$ *passage;* ***C*** *-* $11^{th}$ *passage;* ***D*** *-* $8^{th}$ *passage +* $10^{th}$ *passage, (1:1);* ***F*** *-* $7^{th}$ *passage +* $12^{th}$ *passage (1:1).*

***Statistical Analysis (Student's t-test):-***

[a] *$p<0.001$; significant difference in spleen LPS levels between experiments.*

[b] *$p<0.05$; significant difference in liver LPS levels between experiments.*

[c] *$p>0.05$; no significant difference in liver LPS levels between experiments using same inoculum (C); spleen LPS levels significantly ($p<0.001$) different.*

[d] *Correlation coefficient = 0.49 ($p>0.05$); no significant correlation between liver and spleen LPS levels.*

### 3.6. Discussion

The results described in this chapter illustrate that the LPS-ELISA provides a simple and effective assay for measuring chlamydial infection in murine tissue. Recovery of chlamydial LPS from heat-treated/sonicated tissue correlated well with estimated titres of viable chlamydiae using a cell culture technique. This is consistent with the observation that chlamydial LPS is secreted on to the surface of infected cells (Karimi *et al,* 1989) in increasing quantities as the chlamydiae develop (Campbell *et al*, 1994). Not only were the results obtained considerably faster by LPS-ELISA compared with cell culture, but the mode of detection was also less laborious, replacing microscopic counting of inclusions with spectrophotometry. In contrast to sheep placental tissue where detergent extraction was required to solubilise chlamydial LPS (Jones, unpublished), murine liver was successfully treated by boiling and sonicating in PBS only. The reason for this discrepancy may reflect the exposure of liver cells *in situ* to bile secretions of which sodium deoxycholate is one, thereby increasing the concentration of detergent in the sample. An excessively high concentration of alkaline detergent may firstly affect the LPS structure which normally resides in an acidically charged environment within the outer membrane and secondly, may remove bound material from the microtitre plate.

The standard LPS control used in the ELISA originated from *Salmonella minnnesota* Re, indicating that the monoclonal antibodies used were indeed specific for the epitope shared by *Chlamydia* spp. and other Gram-negative bacteria, (α-2,4-linked disaccharide) (Brade *et al.*, 1985; 86; 90; Nurminen *et al.,* 1983; 84), rather than the genus-specific epitopes unique to *Chlamydia* spp. (α-2,8-linked disaccharide or αKdo-(2,8-αKdo-2,4-αKdo) trisaccharide).

It was also demonstrated that CBA/ADRA mice were exceedingly susceptible to chlamydial infection which could be detected in the liver and spleens of inoculated mice. It was previously shown (Rodolakis *et al*, 1989 ) that the route of injection of inoculum into mice, influenced the spread of infection throughout the body. If the

inoculation strain was of low virulence, then inoculation via the foot-pad could prevent infection progressing beyond the popliteal lymph node. However, intraperitoneal inoculation resulted in even the less virulent intestinal strains becoming systemic and colonising spleen and liver and in pregnant mice could even induce abortion although a much greater (x500) titre of intestinal chlamydiae was required than when abortifacient strains were injected. Similar titres of abortifacient chlamydiae were used in the present study and these produced considerable infection in both the spleen and liver by intraperitoneal injection. Thus, the strain of chlamydia used in the present study was comparable to the abortifacient strains used in the work of Buzoni-Gatel *et al* (1990). In the current study, no significant difference was associated with intravenous injection compared with intraperitoneal injection and both methods resulted in systemic spread of infection. It was hypothesised that the ability of *C. psittaci* to colonise macrophages and thus avoid exposure to the immune system, could explain the systemic nature of ovine chlamydial infection (Wyrick and Brownridge, 1978). In the protocol used in this chapter, peritoneal macrophages may indeed be engaged in dispersal of chlamydial EBs to various organs, including the liver and spleen.

Susceptibility to chlamydial infection in mice is thought to involve both H-2 and non-H-2 related genes. In a pilot study preceding the current work, CBA and CBA/ADRA mice were found to be exceedingly susceptible to chlamydial infection compared with either Balb C or DBA strains of mice (Jones, unpublished). Thus CBA/ADRA mice were chosen for the present study. Rodolakis *et al* (1994) demonstrated the lack of infection in Balb C mice which carried the $H\text{-}2^b$ locus, whilst $H\text{-}2^k$ mice were much more susceptible. This is in agreement with both the pilot study and the data presented here in which CBA/ADRA mice appeared to be highly susceptible to chlamydial infection.

Spleen weights of infected mice were significantly higher than in non-infected mice, both at 4 and 8 days after inoculation, indicating that blastogenesis was probably occurring in response to chlamydial infection (McCafferty *et al.,* 1994). Spleen

weights were significantly higher on day 8 than day 4 which is consistent with long term resolution of infection over weeks rather than days. In addition, by day 8, spleen weights were significantly higher in mice injected with a lower, but still virulent titre of chlamydiae, reflecting possibly less stimulation of suppressive factors which have been shown to act on lymphocytes *in vitro,* e.g. activated splenic macrophages (Tomioka *et al*, 1990) and IFN-γ (Richard *et al,* 1991).

It was interesting that although inoculum titres were standardised between all experiments, varying mean infection levels were detected in mouse tissues between experiments. Peeling and Brunham (1991) reported that the kinetics of infection could be grossly affected by the ratio of dead to live chlamydial particles in the inoculum. Since only viable chlamydiae contribute to the titre in tissue culture, it was unknown what proportions of the inocula were dead chlamydiae. It is possible therefore, that differences in the amounts of chlamydiae recoverable from spleen and liver could have been caused in part, by differing ratios of dead to live chlamydiae between inoculum batches.

# CHAPTER 4

## PROTECTION STUDIES IN MICE USING NATIVE CHLAMYDIAL ANTIGEN

## 4.1. Introduction.

Since the demise of the original chlamydial vaccine (McEwan and Foggie, 1954), and the subsequent increase in abortions due to *C. psittaci,* an alternative vaccine has been sought. In this chapter, the mouse models described in Chapter 3 demonstrating susceptibility of CBA/ADRA mice and detection of chlamydial infection in liver and spleen were applied to protection studies with native *C. psittaci.* Protection was calculated as the percentage difference in chlamydial LPS levels between positive and negative control groups (Jones *et al.,* 1989). Other groups have assessed protection in pregnant mice (Rodolakis, 1983) afforded by murine polyclonal and monoclonal antibodies as the increase in surviving offspring or in non-pregnant mice as a reduction in cultivable chlamydiae from spleen tissue (McCafferty *et al.,* 1994). Reduction in mouse mortality has also been used to indicate a reduction in chlamydial multiplication *in vivo* (Williams *et al.,* 1990). The latter two examples investigated effects due to cytokines rather than vaccines, however, the principles involved in measuring the effects are equivalent.

Both Models 1 and 2 were examined. In the first, the protective effect of a native chlamydial vaccine was directly measured by immunisation of mice. In the second, passive transfer of serum antibodies from sheep which had aborted due to *C. psittaci* infection was assessed with regards to reducing chlamydial LPS levels in mice. Both models relied on *in vitro* analysis (section 3.2.) of organs by LPS-ELISA.

## 4.2. Protection in Model 1 - Active immunisation of mice

The aim in this section was to demonstrate that the infection induced in mice by intraperitoneal injection with live *C. psittaci*, could be reduced to a significant extent by vaccination with an adjuvanted MOMP-enriched preparation. Protection induced by this protocol was compared over 3 experiments in which either the inoculum batch or the adjuvant was altered between experiments.

### 4.2.1. Materials and Methods.

#### 4.2.1.1. Vaccine preparation.

A MOMP-enriched preparation was used as the antigen phase of a native chlamydial vaccine (Materials and Methods, section 2.5.2.). Briefly, *C. psittaci*, strain S26/3, was harvested from tissue culture and semi-purified by the addition of sarkosyl to solubilise the MOMP fraction. The resulting preparation comprised predominantly MOMP, which constituted approximately 25% of the total chlamydial protein. The antigen phase was emulsified (1:1) with either Marcol Arlacel A + Alhydrogel or a second adjuvant, Montanide ISA 50 (+/- Alhydrogel) to form a vaccine. Details of vaccine compositions are given in section 2.5.3.

#### 4.2.1.2. Vaccination protocol.

In the Model 1, mice were immunised with 0.1ml of native chlamydial vaccine on 2 occasions, 3 weeks apart. Immunisation was subcutaneous on both occasions over alternate shoulders. Three weeks after the second vaccination, mice were bled from the right lateral tail vein and the sera analysed for anti-chlamydial antibodies. The following day, each mouse was challenged intraperitoneally with $1x10^6$ ifu/ml *C. psittaci* in 1ml and was killed 6 days later. Results of 3 such experiments are given in Table 4.1., where infection is expressed as levels of chlamydial lipopolysaccharide

in liver and spleen (ng/g) and protection as a percentage compared to the placebo vaccinated controls.

### 4.2.2. Results

#### 4.2.2.1. Reduction in chlamydial LPS levels in mouse tissues.

Protection was assessed as a reduction in chlamydial LPS levels in liver and spleen of mice vaccinated with MOMP enriched antigen, compared to those immunised with placebo vaccine. As in Chapter 3, the variation in LPS levels within a group was extremely high (Table 4.1). This made determination of protection difficult, and consequently the data lacked statistical significance. It was not therefore possible to compare the results of the 3 individual experiments, except to conclude that antigen adjuvanted in Montanide ISA 50 may induce greater protection than Marcol Arlacel A + Alhydrogel.

Several points are exemplified by the tabulated data. Initially, example 3b showed the effect that a single outlying value could have on the data. In this case, a mathematical transformation was required to improve statistical evaluation (Table 4.2). By taking a log function of each value ($\log\{x+1\}$), the distribution was condensed as shown in Fig.4.1. and significance of difference then resolved as $p<0.01$ in the spleen as well as in the liver.

A similar effect was achieved by categorising each individual value in experiment 3b as either positive or negative and applying Fisher's Exact Test (Table 4.2). In this case, the distinction was straight forward due to the high number of negative mice ($x=0$) in the vaccinated group. Only 2/9 (22%) of vaccinated mice were positive for LPS in the spleen, compared to 7/9 (78%) in the placebo group. If, however, the LPS level was not reduced to zero, but rather was a series of declining values as in Experiment 6, it was preferable to calculate a level above which mice could be classed as positive. In this experiment, a placebo vaccinated, non-infected group was

available which allowed a cut-off value of {μ+3SD} to be calculated. Transforming the data with the log{x+1} function had little effect in this case, presumably because of the low LPS values in general. However, using the calculated cut-off value (4.28 ± 2.44; n=10), only 50% of the vaccinated group were positive compared to 85% of the placebo vaccinated group.

It was also apparent from the data (Table 4.1), that the frequency with which negative LPS levels could be expected in a placebo vaccinated, infected group varied considerably depending on the group size. If a low sample size was considered (Experiment 3a; n=3), then the negative animal carried an unnaturally high weighting (approximately 33% of total) and the overall variation was exceedingly large. In contrast, a sample of size 20 was shown only to have a maximum of 15% negative mice.

#### 4.2.2.2. Serological response to vaccination with native chlamydial antigen.

Immunoblotting using sarkosyl-purified chlamydial elementary bodies as antigen, showed a distinct band at approximately 38Kd indicating good seroconversion to the MOMP component of the vaccines (Fig. 4.1.).

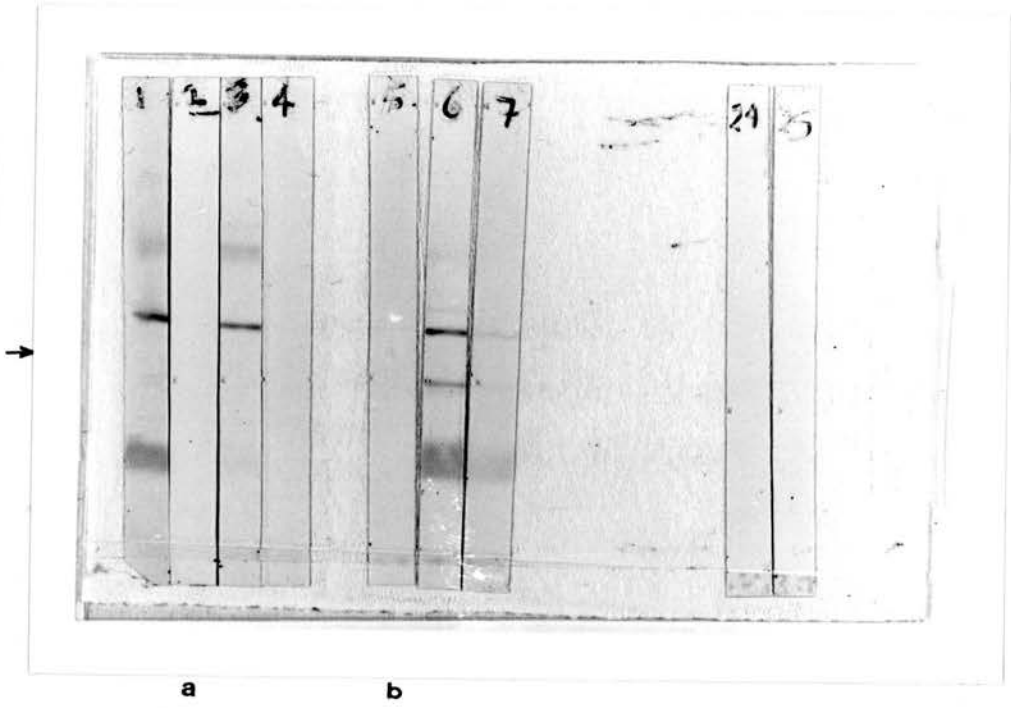



***Fig 4.1.*** *Immunoblot of chlamydial antigen probed with sera from mice immunised with native chlamydial vaccine in adjuvant a) Marcol Arlacel A + Alhydrogel or b) Montanide ISA 50.* (arrow indicates position of MOMP

***Table 4.1.*** *Variation in protection with native chlamydial vaccine.*

| Expt. | *vaccine* | | | *Chlamydial LPS (ng/g)* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Adjuvant | Inoculum | vaccine | spleen | | | | | liver | | | | |
| | | | | **mean** | *sem* | *n* | %P | [1]p< | **mean** | *sem* | n | %P | [1]p< |
| **3a** | M/A+Al. | A | placebo | **1288** | *726* | 3 | | | **6467** | *4042* | 3 | | |
| | | | + | **686** | *211* | 9 | 47 | NS | **1619** | *491* | 9 | 75 | NS |
| **3b** | Mont. | A | placebo | **6091** | *4074* | 6 | | | **16864** | *5717* | 6 | | |
| | | | + | **56** | *41* | 9 | 99 | NS | **202** | *79* | 9 | 99 | 0.01 |
| **6** | Mont.+Al. | D | placebo | **275** | *239* | 20 | | | **12** | *2* | 20 | | |
| | | | + | **29** | *18* | 10 | 89 | NS | **32** | *10* | 10 | - | NS |

*A - 7th passage inoculum; D - 8th + 10th passage inocula, (1:1 ratio).*

- Groups of n mice injected subcutaneously with either placebo or native chlamydial vaccine (+) in adjuvant.
- Adjuvants comprised :- 3a - Marcol Arlacel/A + Alhydrogel; 3b - Montanide ISA 50; 6 - Montanide ISA 50 + Alhydrogel.
- p values calculated by Student's t-test. NS, ($p>0.05$).
- % Protection = $1 - \frac{[\text{LPS in MOMP enriched vaccinates}]}{[\text{LPS in placebo vaccinates}]} \times 100$

***Table 4.2.*** *Reduction in chlamydial LPS levels in mice vaccinated with MOMP enriched preparation; mathematical transformations of data.*

| | | [1]Log {Chlamydial LPS + 1} (ng/g) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | spleen | | | | | liver | | | | |
| **Expt.** | **vaccine** | **mean** | ***sem*** | **n** | **p** | **N° +ve** | **mean** | ***sem*** | **n** | **p** | **N° +ve** |
| **3a** | placebo | **2.18** | *1.09* | [2]3 | | [3]2/3 | **2.63** | *1.32* | [2]3 | | [3]2/3 |
| | + | **2.21** | *0.43* | 9 | NS | [3]7/9 | **2.79** | *0.37* | 9 | NS | [3]8/9 |
| **3b** | placebo | **2.98** | *0.62* | 6 | | [3]5/6 | **3.51** | *0.71* | 6 | | [3]5/6 |
| | + | **0.53** | *0.35* | 9 | p<0.01 | [3]2/9 | **1.39** | *0.44* | 9 | p<0.01 | [3]5/9 |
| **6** | placebo | **1.41** | *0.15* | 20 | | [4]17/20 | **1.04** | *0.05* | 20 | | [4]13/20 |
| | + | **1.11** | *0.16* | 10 | p=0.09 | [4]6/10 | **1.11** | *0.11* | 10 | NS | [4]7/10 |

[1] data transformed by function; *Log{x+1}*. [2] including zero value which contributed abnormally large amount (33%) to group statistics. [3] number of mice positive for LPS (x>0). [4] number of mice positive for LPS (x>mean {non-infected control group} + 3 SD). NS, non-significant.

- Groups of n mice injected subcutaneously with either placebo or native chlamydial vaccine in adjuvant.
- Adjuvants comprised :- 3a - Marcol Arlacel/A + Alhydrogel; 3b - Montanide ISA 50; 6 - Montanide ISA 50 + Alhydrogel.
- p values calculated by Student's t-test.

### 4.2.3. Summary.

LPS levels in the spleen, but not the liver in mice inoculated with $1x10^6$ ifu/ml of *C. psittaci*, strain S26/3, were shown to reach consistent levels when the same batch of inoculum was used in different experiments (Chapter 3). In this chapter, protection against chlamydial multiplication was attempted by immunisation of mice with native chlamydial antigen in 2 different adjuvants. LPS levels in mice injected with placebo vaccine were in the same range for each experiment performed with batch A inoculum (Experiments 3a and 3b), showing no significant differences between mean values ($p>0.05$). However, high variation in LPS levels in all 3 experiments, directly affected the statistical validity of the protective effect of the chlamydial vaccine. None of the results for spleen protection were therefore significant ($p>0.05$). In one such case (Experiment 6- batch D inoculum), LPS levels were low in comparison to batch A inoculum, and whilst 89% protection was achieved, the results were non-significant ($p>0.05$).

Use of an alternative statistical method to the Student's t-test however, allowed a valid assessment of protection to be made based on the percentage positive values in the vaccinated compared to the placebo vaccinated groups. The effect of a large group variation was also reduced in Experiment 3b, by mathematical transformation of the data by $\log\{x+1\}$, resulting in a significant effect ($p<0.01$) of the vaccine. No significant effect could be calculated for Experiment 3a, in which the placebo group contained only 3 members. This highlighted the importance of adequate group numbers in order to validate the data, statistically.

In Chapter 3, a larger range of experiments indicated that over 1/4 (29%) of experiments would be statistically invalid using this model, solely because of high variation within the infected control group. The data presented in this chapter corroborates this, but despite these difficulties appropriate statistical analysis was able to select the adjuvant Montanide ISA 50 for future vaccine studies.

Thus, Model 1 appears to depend on several factors including the effects associated with large group variations, small sample sizes, low inoculum infectivity, outlying values which may require transformation of the data, preferential multiplication of chlamydiae in the spleen and finally the contributing effect of adjuvant to protection.

### 4.3. Protection in Model 2 - Passive immunisation of mice with immune sheep serum.

Whereas Model 1 probably involved both humoral and cellular immune responses after vaccination of mice with MOMP enriched antigen, Model 2 was designed to test the protective capacity of anti-chlamydial antibodies only. Unlike Model 1, where the immune system was stimulated by a MOMP enriched preparation, antibodies used in Model 2 originated from sheep in response to abortion caused by viable chlamydial organisms. In the latter case, sera from ewes which had aborted due to chlamydial infection were pooled and shown to be positive for chlamydia-specific antibodies by ELISA. The protective capacity of this pool of "immune" serum was determined by incubating an equal volume of the serum pool with chlamydial inoculum in order to allow interaction between antibodies and antigen. The mixture was then injected intraperitoneally into mice and infection levels assessed as before, by detection of chlamydial LPS levels in liver and spleen. The importance of complement at the *in vitro* incubation stage was assessed by heat inactivation of the serum and the addition of either heat inactivated or intact guinea pig serum.

#### 4.3.1. Materials and Methods.

Model 2 comprised 2 phases; **Phase 1:-** pregnant adult sheep were infected by s.c. injection with 2 strains of *C. psittaci* (S95/3 and S26/3; $5x10^5$ ifu) at 70-73 days gestation. Blood samples collected approximately 6 weeks after abortion were pooled and tested by ELISA for high chlamydia-specific antibody titre.

**Phase 2:-** equal volumes of pooled "immune" serum (heat inactivated) and chlamydial inoculum were incubated for 45 min. at 37°C with occasional mixing. The mixture contained a final inoculum titre of 2 x $10^6$ ifu/ml and serum concentration of 25%. Each mouse was challenged intraperitoneally with 1ml of mixture and killed 6 days later. Chlamydial infection in the liver and spleen was assessed by capture ELISA of LPS.

#### 4.3.1.1. Requirement for exogenous complement.

Initially, the effect of exogenous complement on the system was examined. *In* vitro neutralisation often requires the presence of complement in the medium. Whilst the mouse strain used in the present study had an intact complement system, the exogenous addition of complement before inoculation may pre-empt endogenous complement and affect neutralisation. Mixtures of heat inactivated immune serum (25% final volume) and chlamydial inoculum (2x$10^6$ ifu/ml final concentration) were incubated as in section 4.3.1., with the addition of either intact or heat inactivated guinea pig serum at a level of 5% (final volume). Naive lambs were also bled and the pooled sera used to form a control serum pool (naive serum) which tested negative for anti-chlamydial antibodies by ELISA.

*Design of experiment to investigate the requirement of exogenous complement for protection in Model 2.*

| group | n | [1]serum source | complement (5% final vol.) |
|---|---|---|---|
| 1 | 7 | immune serum | intact guinea pig serum |
| 2 | 7 | immune serum | [1]H.I. guinea pig serum |
| 3 | 8 | naive serum | [1]H.I. guinea pig serum |

immune serum; standard pool of sera from ewes which had aborted due to *C. psittaci* infection; naive serum; standard pool of sera from naive sheep; [1] H.I., heat inactivated for 30 min at 56°C.

Inoculum mixtures comprised equal volumes of chlamydial inoculum (2 x $10^6$ ifu/ml, final titre) and H.I. sheep serum (25% final volume). Guinea pig serum was added either intact or H.I. at 5% final volume.

#### 4.3.1.2. Variation in protection between experiments.

Several experiments were performed using the procedure described for Model 2, without an additional source of complement. The results for immune and naive sheep serum pools were compared over a range of 9 experiments, in order to assess the degree of variation between experiments carried out on different days and with different inocula. Pools of immune and naive sheep sera remained constant throughout.

### 4.3.2. Results

#### 4.3.2.1. Requirement for exogenous complement.

The result of this study established that an exogenous source of complement was not required. Thus, Group 3 (naive serum), produced significantly higher ($p<0.01$) concentrations of chlamydial LPS in liver samples, compared to either Group 1 or 2 (Table 4.3.). No significant difference ($p>0.05$) was apparent between Group 1 recipients of immune serum/ *C. psittaci*/ complement and Group 2 mice given immune serum/ *C. psittaci*/ H.I. complement. This did not, however, rule out an involvement of complement ***in vivo***. Three mice out of 8 in Group 3 had no detectable chlamydial LPS in either liver or spleen samples. This accounted in part for the high variation seen in Group 3. No LPS was detected in splenic tissue of any of the mice. However analysis of this model in Chapter 3, showed that LPS levels were more reliably detected in the liver on day 6 after infection rather than the spleen and thus the results from the liver were considered sufficient to assess protection in the current experiment.

*Table 4.3. Requirement of Model 2 for exogenous source of complement.*

| Group | n | inoculation mixture | Chlamydial LPS in liver (ng/g) | |
|---|---|---|---|---|
| | | | **mean** | ***sem*** |
| 1 | 7 | [1]immune serum/ *C. psittaci* / intact GPS | **0** | *0* |
| 2 | 7 | [1]immune serum/ *C. psittaci* / H.I. GPS | **0** | *0* |
| 3 | 8 | [1]naive serum/ *C. psittaci* / H.I. GPS | **2961** | *1055* |

Refer to legend attached to Table 4.3.

- [1]All serum aliquots were heat inactivated at 56°C for 30 min. immediately before use. Mixtures comprised equal volumes of chlamydial inoculum (2 x $10^6$ ifu/ml, final titre) and H.I. sheep serum (25% final volume).
- Guinea pig serum was added either intact or heat inactivated at 5% final volume and mixtures incubated 45 min./37°C.
- Each mouse was injected intraperitoneally with 1ml mixture and killed 6 days later. Infection in the liver was assessed by capture ELISA of LPS.

**Statistical analysis (Student's t-test)**

- significant difference between Group 3 and either Group 1 or 2 ($p<0.01$).
- no significant difference between Groups 1 and 2 ($p>0.05$).

#### 4.3.2.2. Reduction in splenic and hepatic chlamydial LPS after passive transfer of immune sheep serum - Model 2.

Immune serum containing a high titre of chlamydia-specific antibodies which were produced in response to abortion, was tested in mice for its protective capacity by incubating with an equal volume of chlamydial inoculum prior to injection intraperitoneally into mice. Table 4.4. indicates the percentage reduction achieved in chlamydial LPS levels in each of 9 experiments. Standard immune sheep serum caused a 81-100% reduction of LPS in the liver, with a consistently high degree of significance over the 9 experiments ($p<0.01$; 7/9 experiments). In contrast, levels of reduction ranging from 72-100% were noted in the spleen and 2/8 experiments were not significant ($p>0.05$) and only 3/8 reached a highly significant level ($p<0.01$). The variation in reduction of LPS was, as described in Chapter 3, reduced by arranging experiments into blocks according to the inoculum used. With this arrangement, LPS reduction in the liver ranged from 91-100% incorporating inocula A, C and D and 99-100% where inocula A and D only were considered.

### 4.3.3. Summary.

In general, the reduction in LPS levels in the liver was statistically more significant than in the spleen. This reflects the findings of the previous chapter, where LPS levels in the liver were more consistent between animals of the same group than in the spleen. In this case, there was no apparent need to mathematically transform the data in order to reduce the variation between groups. Not only was variation in general lower in this model than in Model 1, but reduction in LPS levels by immune serum was sufficient to yield significant protection. It is worth considering, however, that the purpose of the model was to test potential vaccine antigens which would not be expected to induce such high seroconversion as was present in serum from sheep recovering from an acute infection. This being the case, it proved necessary to employ other statistical applications to data in later chapters, such as Fisher's test or mathematical transformations, in order to highlight differences between vaccines.

The above results, however, demonstrated that immune serum taken from sheep 6-8 weeks after aborting, was able to significantly reduce chlamydial LPS levels in mouse liver and spleen tissue, thus confirming a role for humoral immunity in protection against *C. psittaci* infection.

Legend for Table 4.4.

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**A** - $7^{th}$ passage; **B** - $8^{th}$ passage; **C** - $11^{th}$ passage; **D** - $8^{th}$ passage + $10^{th}$ passage (1:1); **F** - $7^{th}$ passage + $10^{th}$ passage, (1:1).-, naive serum; +, immune serum; **ND**, not determined; **NS**, not statistically significant ($p>0.05$).

- Live chlamydiae ($2x10^6$ ifu/ml, titre at injection) incubated at 37°C/45min. with an equal volume of H.I. sheep immune (+) or naive (-) serum.
- 8-14 week old mice inoculated intraperitoneally with 1ml mixture.

  mice killed on day 6 p.i.; infection of spleen and liver assayed by capture ELISA of chlamydial LPS.
- % Protection (% P) = 1 - [ LPS in immune serum group ] x 100

  [ LPS in naive serum group ]
- Statistical analysis by Student's t-test ($p<$).

---

***Table 4.4.*** *Reduction in splenic and hepatic chlamydial LPS levels after passive transfer of immune sheep serum - Model 2.*

| | | spleen | | | liver | | |
|---|---|---|---|---|---|---|---|
| **Expt./ Inoculum/ serum** | **n** | **mean LPS (ng/g)±sem** | **% P** | **p<** | **mean LPS (ng/g)±sem** | **% P** | **p<** |
| 8/A/- | 8 | 840 ± 152 | 100 | 0.01 | 3259 ± 1423 | 99 | 0.05 |
| + | 8 | 0 ± 0 | | | 33 ± 16 | | |
| 14/A/- | •12 | ND | ND | ND | 494 ± 146 | 100 | 0.01 |
| + | •12 | ND | | | 0 ± 0 | | |
| 16/A/- | •10 | 76 ± 42 | 100 | NS | 296 ± 106 | 100 | 0.01 |
| + | •7 | 0 ± 0 | | | 0 ± 0 | | |
| 10/B/- | 7 | 249 ± 79 | 79 | 0.05 | 367 ± 134 | 81 | 0.05 |
| + | 9 | 53 ± 9 | | | 71 ± 21 | | |
| 18/C/- | 9 | 950 ± 52 | 99 | 0.01 | 347 ± 44 | 100 | 0.01 |
| + | 9 | 13 ± 4 | | | 0 ± 0 | | |
| 20/C/- | 10 | 648 ± 128 | 83 | 0.01 | 1264 ± 160 | 98 | 0.01 |
| + | 10 | 109 ± 73 | | | 26 ± 3 | | |
| 22/C/- | 4 | 2244 ± 445 | 85 | 0.05 | 1000 ± 241 | 91 | 0.01 |
| + | 4 | 281 ± 50 | | | 93 ± 8 | | |
| 7/D/- | 10 | 82 ± 97 | 99 | NS | 378 ± 134 | 100 | 0.01 |
| + | 15 | 1 ± 1 | | | 0 ± 0 | | |
| 19/F/- | 5 | 1703 ± 275 | 72 | 0.05 | 2280 ± 497 | 82 | 0.01 |
| + | 7 | 479 ± 244 | | | 405 ± 79 | | |

- See previous page for legend

## 4.4. Discussion.

Two murine models were developed in which chlamydial infection could be measured as chlamydial LPS content of spleen and liver tissue. In the first model mice were injected subcutaneously with native antigen prepared as a MOMP-enriched fraction. CBA/ADRA mice seroconverted strongly to MOMP when vaccinated with this preparation adjuvanted in Montanide ISA 50 and were subsequently protected from chlamydial infection. The contributions of cell-mediated and humoral immune responses to protection were not determined, however, both systems have been demonstrated to function simultaneously, although cellular immunity may be the more dominant (Buzoni-Gatel *et al.*, 1992).

In the present study, passive transfer of immune serum resulted in greater protection against chlamydial infection than did immunisation with a MOMP-enriched vaccine. It is possible that the high titre of the immune serum was responsible for the observed effect and that if serum from vaccinated ewes had been used instead, the protective effect would not have been so absolute. For further experiments, however, it was useful to include the current immune serum pool as a control to ensure that lack of protection was not due to a fault in the model.

In addition, it has been shown that both susceptibility of mice to chlamydial infection (Tuffrey *et al,* 1992) and production of antibodies (Zhong and Brunham, 1992; Westbay *et al*, 1994) are under genetic control. In particular, Buzoni-Gatel *et al* (1994) demonstrated that infection of mouse spleens varied according to both the H-2 locus and the genetic background of each strain. Mice carrying the H-$2^k$ locus such as the CBA strain, were highly susceptible to infection as was the case in the present study. Furthermore, some strains of mice, differing at the H-2 locus produced varying antibody responses against chlamydial MOMP (Westbay *et al*, 1994) and against the heat shock proteins, hsp60 and hsp 70 (Zhong and Brunham, 1992). Again the H-2 locus was investigated and antibody responses against chlamydial

MOMP were shown to be low in mice carrying the H-$2^k$ haplotype. Since a good MOMP-specific antibody response was detected by immunoblotting in the current work, the haplotype of the mice used here is likely to be different from the parent strain of CBA.

Whilst infection levels in control animals were comparable between the models, Model 2 appeared to result in less variation within a group than Model 1, resulting in stronger statistical analysis. Variations in infection levels were no higher than other groups had found. The lower variation obtained between mice in Model 2 could have been due to the use of pooled sera which would eliminate variation due to individual sheep responses to vaccination and essentially result in an indication of the average protective antibody response in sheep. In contrast, Model 1 had at least two opportunities to show variation between animals a) in immune response to vaccination and b) in susceptibility to infection. In addition, opsonisation of chlamydiae with naive sheep serum may have increased the rate of uptake into host cells via FcγRIII receptors in comparison to chlamydiae which had not been pre-opsonised (Model 1). This could have resulted in less exposure of chlamydiae to the immune system, in particular to polymorphonuclear leukocytes which have been shown to have an important role in the early control of intraperitoneal infection by chlamydiae (Barteneva *et al*, 1996). Differences in infection levels may therefore represent a difference in either neutrophil response or chlamydial internalisation by macrophages in individual mice.

The protocol used in Model 1 indicated that Montanide ISA 50 was a suitable adjuvant in which to formulate native chlamydial antigen in order to elicit protection in mice against chlamydial infection. Seroconversion occurred against the vaccine antigen (MOMP-enriched preparation), in particular against the MOMP, indicating that the strain of mouse used in the present study was able to recognise MOMP in its native conformation and respond with a strong antibody response. T-cell help has been documented between MOMP and Omp 2, whereby priming with MOMP results in an amenestic response towards Omp 2 (Westbay *et al.*, 1994; Allen and Stephens,

1993). Evidence for the reverse is limited, however, such events could have important connotations on the effects of mice to a sub-unit or recombinant vaccine in which only one of these domains is present.

The following chapter examines the mode of action of protection *in vivo* by Model 2, considering the early events occurring in the peritoneal cavity immediately after injection with inoculation mixture.

# CHAPTER 5

## KINETICS OF *IN VIVO* NEUTRALISATION

## 5.1. Introduction

The previous chapter showed that Model 2, passive immunisation of mice, represented an efficient model by which serum containing protective antibodies could be identified. Briefly, immune sheep serum was able to reduce chlamydial LPS levels in mouse spleen and liver when incubated with an equal volume of chlamydial inoculum prior to intraperitoneal injection. In this chapter, local immune responses occurring at the site of injection of the inoculum/serum mixture were investigated.

The mode of chlamydial neutralisation *in vivo* remains unresolved. The original work of Friis (1972) and others (Byrne, 1976; Byrne and Moulder, 1978; Wyrick and Brownridge, 1978; Brownridge and Wyrick, 1979) demonstrated that internalisation of chlamydiae was induced by the organism itself and not by the host cells and that the survival of chlamydiae within host cells occurred by avoidance of phagolysosomal fusion. Both these events were attributed to surface components on the elementary body (Levy and Moulder, 1982; Eissenberg *et al.*, 1983). Uptake of chlamydiae was shown to occur by two possible mechanisms: by non-clathrin coated pit entry (Kihlstrom and Soderlund, 1983) and alternatively by receptor-mediated endocytosis via clathrin-coated pits (Hodinka *et al.*, 1988; Reynolds and Pearce, 1990). Both modes of entry, however, resulted in the same internal destination for ingested particles (Hansen *et al.*, 1993). Heat-inactivation of chlamydiae, however, was unable to prevent phagolysosomal fusion (Friis, 1972), and acidification and destruction of chlamydia-laden vesicles was completed within 30 mins in macrophages (Eissenberg *et al.*, 1983) and 2h in epithelial cells *in vitro* (Schramm *et al.*, 1996). Only heat-inactivated inclusions were shown to be directed to vacuoles containing lysosomal enzyme (Anderson *et al.*, 1984), further indicating that the intracellular fate of chlamydiae is controlled by surface-exposed components.

Serum from ewes recovering from an acute chlamydial infection reacted strongly with MOMP by immunoblotting. Furthermore, opsonisation of chlamydial

particles with immune sheep serum reduced multiplication of viable chlamydiae *in vivo* using the protocol of Model 2 (Chapter 4). However, it was unknown whether 'neutralisation' of chlamydial infectivity occurred in the peritoneal cavity i.e. at the site of injection, and which cells in a mixed population were responsible for the destruction of chlamydiae. Thus, the current chapter sought to identify the stage at which development of infectious chlamydiae was arrested and to compare the events leading up to destruction of chlamydiae coated with immune sheep serum, with destruction of heat-killed chlamydiae and with survival of chlamydiae opsonised with naive sheep serum.

## 5.2. Materials and Methods.

A serial kill experiment was performed using the protocol for Model 2, in which sheep serum was used to passively immunise CBA/ADRA mice. Four groups were included in the experiment as follows:-

Group 1 - *C. psittaci* + immune sheep serum
Group 2 - *C. psittaci* + naive sheep serum
Group 3 - heat inactivated *C. psittaci* + naive sheep serum
Group 4 - L929 cells + naive sheep serum

Group 4 was included as a control to show that the observed responses were due to *C. psittaci* and not to the presence of other cells in the original culture. Additionally, Group 3 represented a control to show that the observed responses were due to viable *C. psittaci* and could not be duplicated by dead organisms.

At necropsy, peritoneal lavages were taken as described in Materials and Methods (Section 2.8.3.). Each sample of peritoneal exudate was divided between 4 different assays:-

a) total cell count
b) Leishman's differential cell count
c) *chlamydia*-specific immunoperoxidase staining
d) LPS-ELISA

Liver and spleen samples were also taken for LPS-ELISA analysis.

### 5.2.1. Design of experiment

**Table 5.1** *Experimental Design*

| | Number of mice killed (hours p.i.) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Group** | **0** | **3** | **8** | **24** | **48** | **72** | **120** | **144** | **240** |
| **1** | | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| **2** | | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| **3** | | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| **4** | 4 | | | | 4 | | 4 | 4 | 4 |

Group 1; *C. psittaci* and immune sheep serum.

Group 2; *C. psittaci* and naive sheep serum.

Group 3; Heat inactivated *C. psittaci* and naive sheep serum.

Group 4; L929 cells and naive sheep serum.

- *12 week old mice were injected intraperitoneally with 1ml of chlamydial inoculum/serum mixture (0h).*
- *Peritoneal lavages were taken from 4 mice per sampling (hours post-infection, p.i.) for analyses of total cell counts, differential cell counts, immunoperoxidase staining and chlamydial LPS content.*
- *Cytospins were stained with Leishman's stain and the number of individual cells in approximately 10 fields of view (≅ 500 cells) calculated.*
- *Liver and spleen samples removed for LPS-ELISA analysis.*

## 5.3. Results.

The purpose of this experiment was to investigate the cells involved in host defence against *C. psittaci* infection at the site of injection, and to compare the cell population profiles in the presence of immune and naive sera. Briefly, chlamydial inoculum pre-incubated with either sheep immune or naive serum was injected intraperitoneally into groups of mice. Peritoneal lavages were taken over the following 210h and the total number of cells per ml counted as well as the number of lymphocytes, neutrophils, monocyte/macrophages and mast cells per ml. Viability of the inoculum was determined by immunoperoxidase staining of peritoneal cells to show the development of chlamydial inclusions and by capture ELISA of LPS in the peritoneal cells, spleen and liver.

### 5.3.1. Differential response of cells in the peritoneal cavity

Total cell counts were made on a Neubauer counting chamber using trypan blue exclusion. Cytospins were stained with Leishman's stain and the percentages of each cell population counted for approximately 500 cells over at least 10 different fields of view. The two parameters were combined to estimate the number of specific cell types (cells/ml). Cells were divided into 4 predominant populations in the peritoneal exudates; polymorphonuclear leukocytes (PMNLs), monocyte/macrophages (MΦ), lymphocytes and mast cells. Other cell types which may have been present occurred so infrequently that total numbers were not recorded.

#### 5.3.1.1. Total cell counts

Mice in all groups had similar numbers of cells in the peritoneal cavity over the first 48h post infection (p.i.) (Fig 5.1); approximately $2.8 \times 10^6$ cells/ml (sem$<5.0 \times 10^4$ between groups). This was unchanged over the duration of the experiment for Groups 2, 3 and 4. However, there was a significant increase ($p<0.05$) in the total cell population at 72h in group 1 which was more pronounced , increasing by at least ten-fold ($p<0.01$) at 120h. The results of the differential cell counts are discussed below (Fig 5.2-5.5).

#### 5.3.1.2. Polymorphonuclear leukocytes

At 3h post-injection (p.i.), an acute inflammatory response was apparent in Groups 1, 2 and 3 with a significant ($p<0.001$) influx of polymorphonuclear leukocytes (PMNLs) into the peritoneal cavity compared to Group 4 (Fig 5.2). A significant difference ($p<0.05$) was also calculated between Groups 1 and 3 at this time, with a higher number of PMNLs present in Group 3 than 1. The neutrophil response persisted over the following 24h p.i. with no significant difference between the three infected groups ($p>0.05$). Gradually, PMNL counts decreased towards control levels. Group 2 showed the slowest decline in numbers of PMNLs, with control levels not being reached until 72 h p.i. compared with 48h p.i. in Groups 1 and 3 (48h; Group 2 significantly higher than Groups 1 and 3,$p<0.01$). A second influx of neutrophils was observed at 120h p.i. in Group 1 only ($p<0.05$). All other groups remained at baseline levels.

#### 5.3.1.3. Monocytes/Macrophages

Both cell types in this category originate from the same precursor and represent different stages of cell development and activation. Since it was not possible to make a definitive distinction between cells at different developmental stages, monocytes and macrophage cells (MΦ) were counted together.

Initially, mice in Groups 1-3 had significantly ($p<0.05$) fewer MΦ compared to a control value of approximately $2x10^6$ cells/ml in Group 4 (Fig 5.3.). Over the following 24h p.i., the number of MΦ in each group approached that seen in Group 4 with no significant difference between any of the groups ($p>0.05$). However, by 48h p.i., mice in Group 2 again had a significantly ($p<0.05$) lower number of MΦ in the peritoneal cavity than mice in either Group 1 or 4. In contrast, MΦ numbers at 48h p.i. were higher in mice in Group 1 than in Group 4 and continued with this trend up

to 120h p.i. The observed increases were significant ($p<0.01$) at both 72 and 120h p.i.

#### 5.3.1.4. Lymphocytes

There were no significant differences between Groups 1-4 over the first 24h p.i. ($p>0.05$) with respect to lymphocyte numbers in the peritoneal cavity (Fig 5.4.). However, at 120h p.i., mice in Group 1 had a large and significant increase ($p<0.01$) in lymphocyte numbers compared to other sampling times. In contrast, Group 2 mice had a significantly ($p<0.05$) lower number of lymphocytes at these times (48h and 120h p.i.). Clearly, a strong divergence in lymphocyte response between Groups 1 and 2 had developed by day 5 p.i..

#### 5.3.1.5. Mast cells

These cells did not feature significantly in response to inoculation with any of the reaction mixtures. No group showed any significant difference ($p>0.05$) from the control group which itself contained only 1-2% of the total cell number as mast cells (Fig 5.5.). The only significant ($p<0.05$) increase in this cell population occurred in Group 1 at 5d p.i..

#### 5.3.1.6 Summary.

Total cell counts did not differ significantly ($p>0.05$) between the baseline value (Group 4) and Groups 1-3, except in Group 1 at 72h and 120h p.i. ($p<0.05$, $p<0.01$, respectively). However, the initial response to injection was a significant increase in PMNLs ($p<0.001$). In order to maintain a constant total cell count in the peritoneal cavity, the concentration of other cell populations would be expected to decrease. This was seen with the population of MΦ, where levels were significantly ($p<0.05$) lower than the control value (Group 4) in all infected groups.

The significant increase in total number of cells which occurred at 72h and 120h in Group 1, could be attributed to fluctuations in the numbers of 2 cell populations; at 72h p.i., numbers of MΦ significantly increased ($p<0.05$), followed by an increase in the number of lymphocytes at 120h p.i. ($p<0.01$). In contrast, although not large enough to exert a significant effect on overall cell numbers, Group 2 demonstrated a significant decrease ($p<0.05$) in lymphocyte numbers at 120h p.i.. The MΦ population was also depressed in Group 2, in this case at 48h p.i. ($p<0.05$).

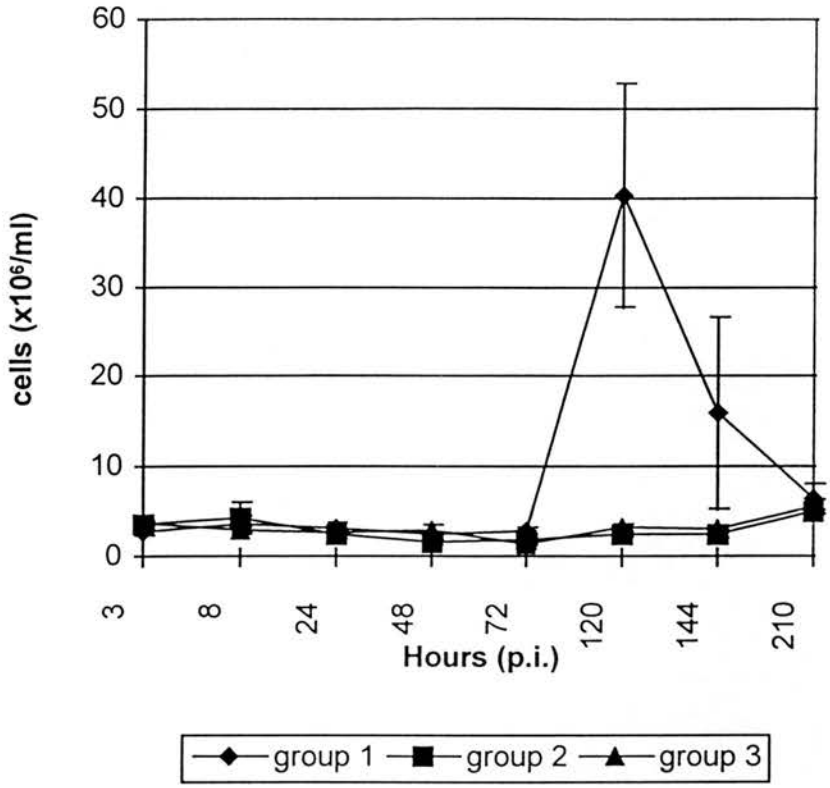


**Fig** ***5.1.*** *Total number of cells per ml in the peritoneal cavities of mice after intraperitoneal inoculation with* Chlamydia psittaci.

**Statistical analysis (Student's t-test) :-**

Number of cells in peritoneal lavages differed significantly in the following cases:-

At 72h p.i. - Group 1 > Groups 2 or 3 ($p<0.05$).

At 120h p.i. - Group 1 > Groups 2, 3 or 4 ($p<0.01$).

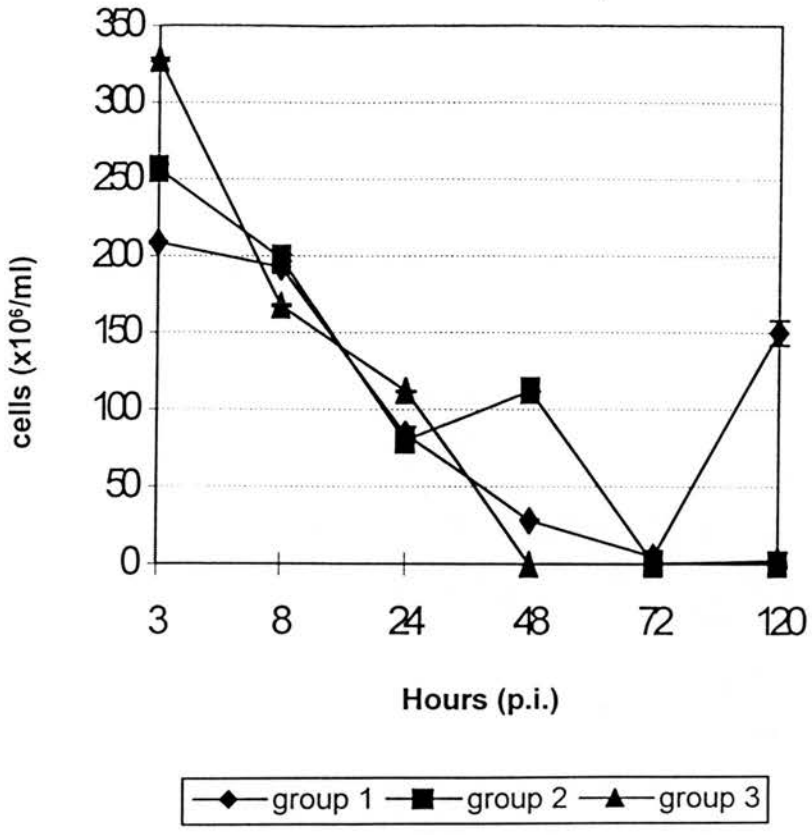


*Fig 5.2. Mean number of polymorphonuclear leukocytes (PMNLs) per ml in the peritoneal cavities of mice after intraperitoneal inoculation with* Chlamydia psittaci.

Refer to Table 5.1 for legend

**Statistical analysis (Student's t-test) :-**

Number of cells in peritoneal lavages differed significantly in the following cases:-

At 3h p.i. - Groups 1, 2 and 3 > control ($p<0.001$).

Group 3 > Group 1 ($p<0.05$).

At 48h p.i. - Group 2 > Groups 1 and 3 ($p<0.01$).

At 120h p.i. - Group 1 > Groups 2, 3 and control ($p<0.05$)

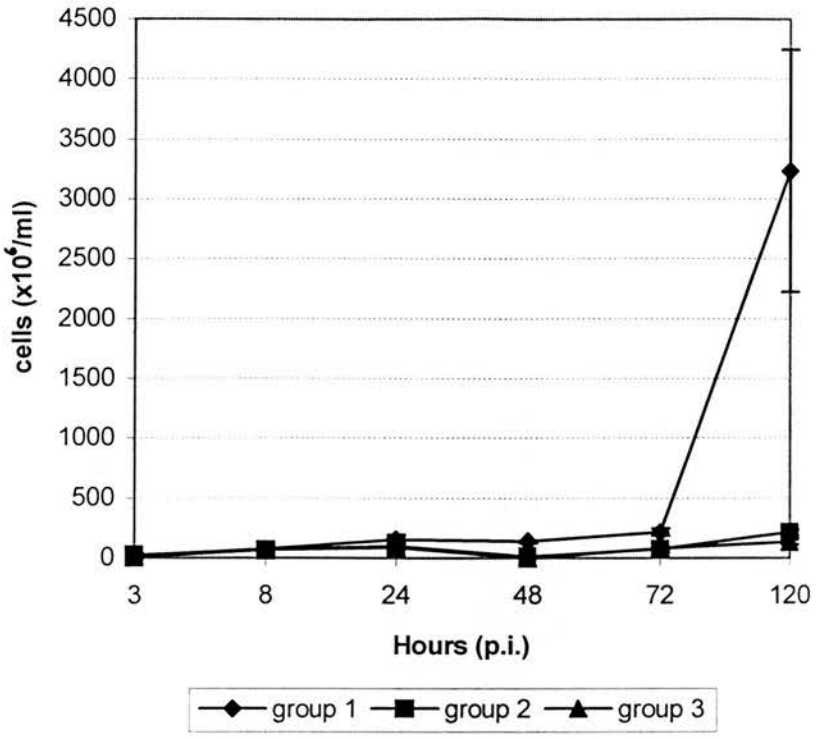


***Fig 5.3.*** *Mean number of Monocyte/Macrophage cells (MΦ) per ml in the peritoneal cavities of mice after intraperitoneal inoculation with* Chlamydia psittaci.

Refer to Table 5.1 for legend

**Statistical analysis (Student's t-test) :-**

Number of cells in peritoneal lavages differed significantly in the following cases:-

At 3h p.i. - Groups 1, 2 and 3 < control ($p<0.05$).

At 48h p.i. - Group 2 < Groups 1 and control ($p<0.05$).

At 120h p.i. - Group 1 > Groups 2, 3 and control ($p<0.01$)

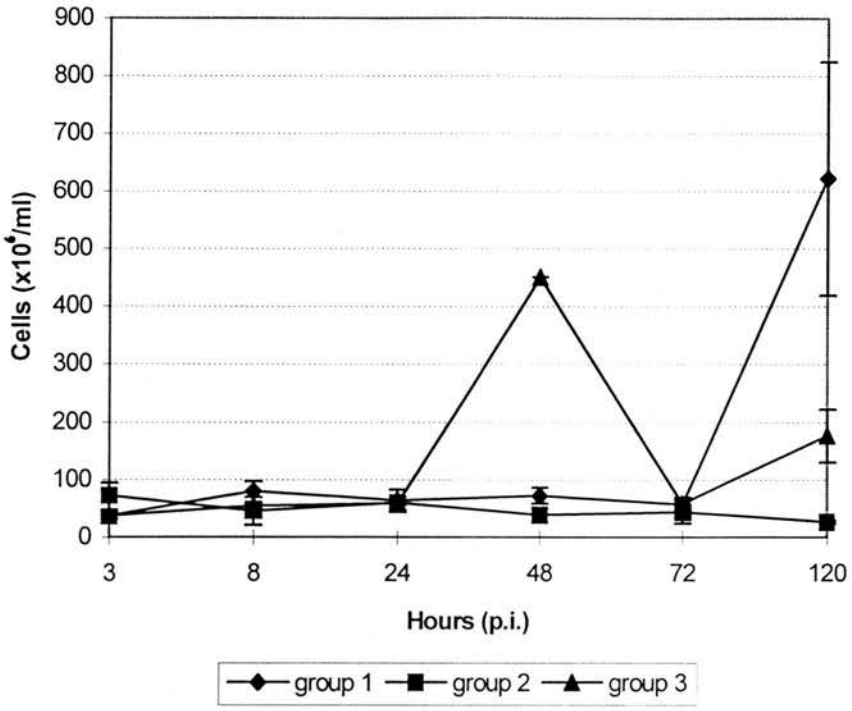


***Fig 5.4.*** *Mean number of Lymphocytes per ml in the peritoneal cavities of mice after intraperitoneal inoculation with* Chlamydia psittaci.

Refer to Table 5.1 for legend

**Statistical analysis (Student's t-test) :-**

Number of cells in peritoneal lavages differed significantly in the following cases:-

At 48h p.i. - Group 2 < control ($p<0.05$).

Group 3 < Groups 1, 2 and control ($p<0.05$)

At 120h p.i. - Group 1 > Groups 2, 3 and control ($p<0.01$)

Group 2 < control ($p<0.05$)

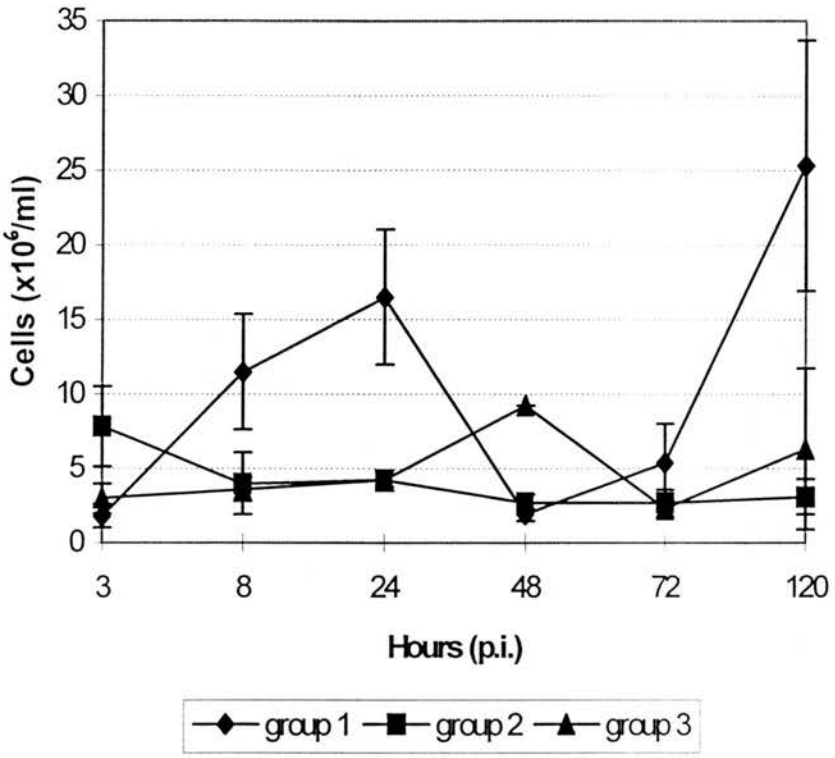


*Fig 5.5. Mean number of Mast cells per ml in the peritoneal cavities of mice after intraperitoneal inoculation with* Chlamydia psittaci.

Refer to Table 5.1 for legend

**Statistical analysis (Student's t-test) :-**

Number of cells in peritoneal lavages differed significantly in the following cases:-

At 120h p.i. - Group 1 > Groups 2, 3 and control ($p<0.05$)

### 5.3.2. Characterisation of peritoneal cells infected with *C. psittaci*

#### 5.3.2.1. Materials and Methods.

*Chlamydia*-specific, immunocytochemical staining was performed on peritoneal cell cytospins. Primary antibody comprised *chlamydia*-positive sheep serum, semi-purified by ammonium sulphate precipitation. Secondary antibody comprised (donkey) anti-sheep IgG conjugated to horseradish peroxidase (HRP). No staining was evident with Group 4 which contained sheep serum but no *Chlamydia*. Likewise, controls comprising a) no primary antibody b) no secondary antibody conjugate c) DAB only, were negative, indicating the specificity of the staining technique for chlamydiae.

#### 5.3.2.2. Results.

Two questions were being asked in this section. Initially, immunoperoxidase staining was used to identify which cells had ingested chlamydiae after intraperitoneal injection of mice and secondly, whether chlamydial inclusions were able to develop in peritoneal cells after pre-incubation with immune serum.

##### 5.3.2.2.1. 3h post infection.

At the first sampling of peritoneal cells post-infection, only cells in Group 3 stained positively for chlamydial antigen indicating rapid uptake of heat-inactivated chlamydiae after injection. The main cell population identified as positive was PMNL, which was the predominant cell type at this time. No positive staining was evident in cells of either Groups 1 or 2.

#### 5.3.2.2.2. 8h post infection.

At 8h p.i. there was still no staining visible in any of the cells in Group 2. In contrast, however, there was now obvious positive staining inside both MΦ and PMNLs in Group 1. Again, there appeared to be greater staining of PMNLs than of MΦ within the peritoneal cavity in this group. The positive staining in cells of Group 3 at 3h p.i. had now disappeared and no internal staining of cells was visible in this group.

#### 5.3.2.2.3. 24h post infection.

As above, no staining was visible in cells of Group 2 and although some staining of MΦ was apparent in Group 1, this was less than was seen at 8h p.i.. PMNLs did not appear to be positive any longer in Group 1. Cells in Group 3 again gave a negative result.

#### 5.3.2.2.4. 48h post infection.

A sharp contrast was seen between Groups 1 and 2 at 48h p.i.. Small amounts of positive staining were visible within MΦ in Group 1, however, much denser, granular and more frequent staining of MΦ, was seen in Group 2 similar to the inclusion bodies seen within infected tissue-culture cells. Some PMNLs also demonstrated positive staining, although these were not the main cells stained due to their comparatively low numbers at this time. Cells in Group 3 continued to remain negative.

#### 5.3.2.2.5. 72h post infection.

By 3 days p.i., large inclusions were visible within MΦ in Group 2. Dense areas of staining were apparent within the cytoplasm, thinning out as the cytoplasm passed over the MΦ nucleus. No staining occurred in cells of either Groups 1 or 3.

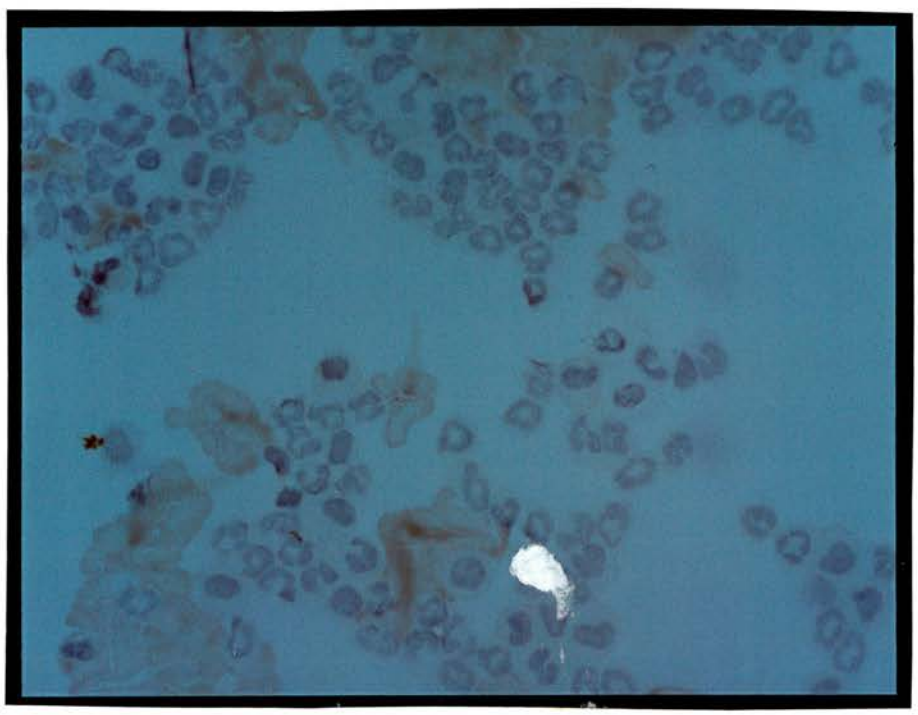

**Fig 5.6.** Immunoperoxidase staining of peritoneal cells at 0 hours (x360 magnification)

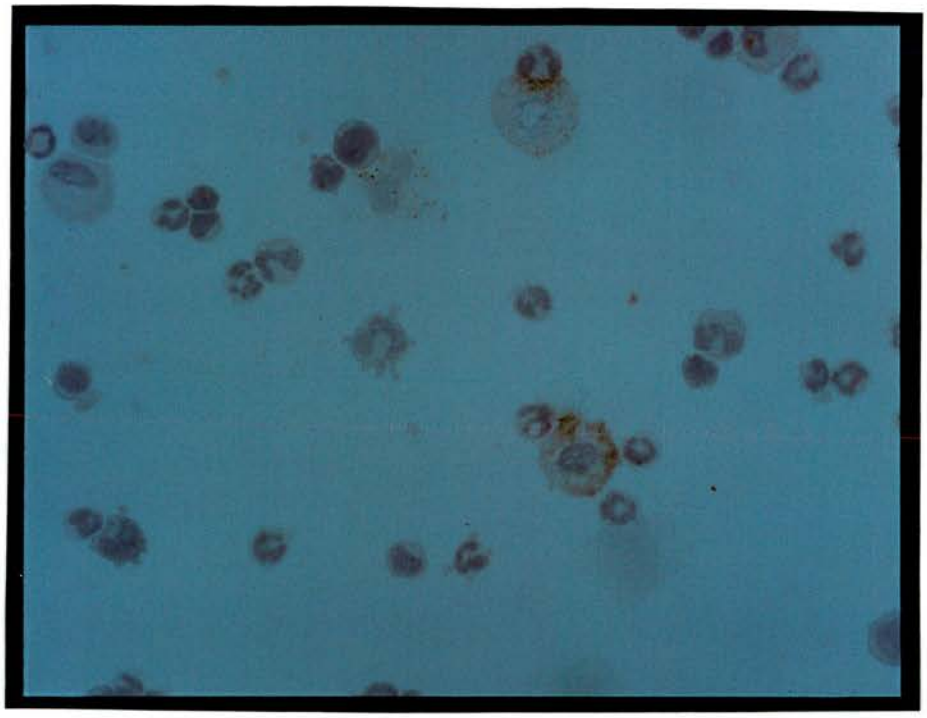

**Fig 5.7.** Immunoperoxidase staining of infected peritoneal cells at 48 hours post infection (x360 magnification)

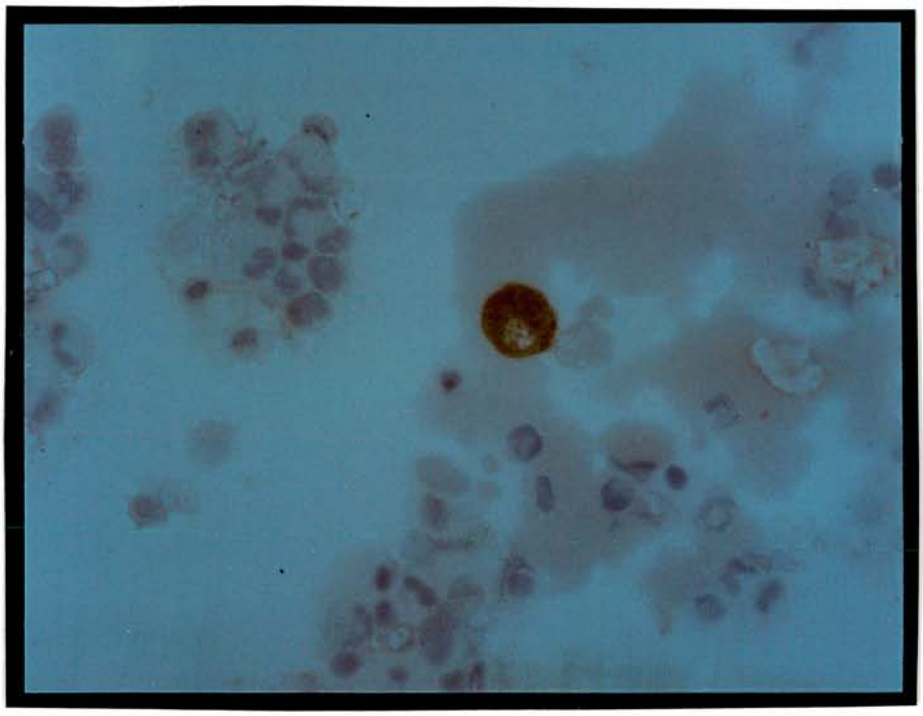

**Fig 5.8.** Immunoperoxidase staining of infected peritoneal cells at 72 hours post infection (x360 magnification)

#### 5.3.2.3. Summary.

Immunoperoxidase staining enabled progress of the chlamydial inoculum to be followed after intraperitoneal injection. This highlighted clear differences between the fate of *C. psittaci* pre-incubated with either immune or naive sheep sera. In the first case, chlamydiae incubated with immune serum appeared to be taken up by cells more slowly than heat-inactivated *C. psittaci*, which was visible after 3h in comparison to 8h with the addition of immune serum to the reaction mixture. By 8h p.i., heat-inactivated *C. psittaci* had disappeared from peritoneal cells. In contrast, inoculum containing immune serum was visible up to 48h p.i., albeit in decreasing amounts. The presence of naive serum in live inoculum however, appeared to delay the detection of *C. psittaci* inside cells until the organism had formed large inclusion bodies, (48h p.i.). This granular staining was also visible at 72h p.i., when the size of the stained inclusion body appeared greater than at 48h.

### 5.3.3. Concentrations of chlamydiae in the peritoneal cavity

This analysis was carried out to confirm the results of the immunoperoxidase staining, by demonstrating that even though chlamydiae would be expected to disperse from the site of injection, multiplication of remaining organisms could be detected by increasing amounts of chlamydial LPS in the peritoneal cavity. "Neutralisation" of chlamydiae at this stage would not result in increased LPS levels.

#### 5.3.3.1. Materials and Methods.

The LPS-ELISA was used to quantify the amount of Chlamydial LPS in the peritoneal cavity for each mouse. Peritoneal fluid was diluted 1/2 in PBS/tween and all samples tested in duplicate following the standard procedure.

#### 5.3.3.2. Results.

An initial peak of chlamydial LPS was detected at 3h p.i. in all mice except those in Group 4 which did not receive any chlamydiae (data not shown). Subsequent samplings revealed continuously higher levels of chlamydial LPS in mice in Group 2 than in mice in either of the other infected groups. At 6 days p.i., only samples from mice in Group 2 had raised chlamydial LPS levels, in parallel with high infection levels in the liver and spleen (see next section).

### 5.3.4. Appearance of infection in internal organs

The purpose of this section was to demonstrate that the chlamydial inoculum used throughout the experiment (not heat inactivated) was viable in the presence of heat inactivated naive sheep serum and that the lack of apparent infection in the liver or spleen was due to the presence of sheep immune serum.

#### 5.3.4.1. Materials and Methods.

The LPS-ELISA was used to quantify the amount of Chlamydial LPS in the liver and spleen for each mouse. Organ samples were treated as described in Chapter 3, diluted 1/5 in PBS/tween, and tested in duplicate following the standard LPS-ELISA procedure (section 2.7.4.).

#### 5.3.4.2. Results.

No chlamydial LPS was detected in significant quantities in either the liver or spleen of mice in any group until day 2 p.i. (data not shown). At this point, a significant ($p<0.001$) amount of chlamydial LPS was detected in the spleen of Group 2 mice. A small increase in chlamydial LPS was also detected in the spleens of Group 1 mice, however, the levels were not statistically significant ($p>0.05$). From 48h onwards, the levels of chlamydial LPS in splenic tissue continued to rise in mice in Group 2.

At 5 days p.i., the quantities were significantly higher ($p<0.001$) than in mice in Group 1.

Infection of the liver showed a similar profile to splenic infection. Establishment of colonisation, however, appeared to be delayed by approximately 24h compared to the spleen. Only Group 2 mice had any significant ($p<0.001$) levels of chlamydial LPS, which were detectable on day 3 p.i. in the liver and continued to rise over the following 3 days. On day 6 p.i., liver colonisation by chlamydiae was approximately half that of the spleen in mice in Group 2.

## 5.4. Discussion

The initial response of mice to peritoneal injection with chlamydiae was a significant influx of PMNLs into the peritoneal cavity. This did not depend on chlamydiae being in a viable state, since H.I. inoculum induced an equivalent response to intact chlamydiae. These results are in agreement with those of Barteneva *et al.* (1996) and others (Brunham *et al.*, 1984; Pal *et al.*, 1993) who demonstrated that the predominant cells recruited to the initial site of infection were PMNLs. It can be speculated that the mediator of the inflammatory response was the heat-stable LPS component on the chlamydial surface, since the early samplings (3h) would not have allowed sufficient time for *de novo* synthesis of other possible stimulants. Ingalls *et al.*, (1995) also suggested a major role for LPS in the acute inflammatory reaction demonstrating the induction of TNF-α *in vitro* in response to stimulation with chlamydial LPS. They also showed that chlamydial LPS was approximately 100-fold less potent than the LPS of either *S. minnesota* or *N. gonorrhoeae* and could only be classed as a weak endotoxin. Thus, whilst PMNLs may help to control an initial chlamydial infection, other immune responses probably supercede the inflammatory response.

As the levels of PMNLs decreased with time, it was observed that higher levels persisted in mice injected with viable chlamydiae in naive sheep serum (Group 2) compared with immune serum or heat killed chlamydiae. This may represent a continuing stimulation of the inflammatory response by endotoxin, which implies that sufficient antigen was available only in Group 2 mice. Campbell *et al.*, (1994) demonstrated the secretion of LPS by developing chlamydiae on to the host cell surface and it was perhaps this phenomenon which caused the continuing stimulation of the PMNL response. When levels of macrophages and lymphocytes in the peritoneal cavity were analysed, it was discovered that both cell populations were present in lesser amounts in mice injected with viable chlamydiae in naive serum compared to mice in other infected groups. A complex sequence of immune responses is thought to occur against infectious chlamydiae, importantly the secretion

of IFN-γ which is a major cytokine involved in limiting chlamydial replication (McCafferty *et al.*, 1994). IFN-γ has been shown to effectively suppress lymphocyte proliferation *in vitro* (Richard *et al.*, 1991) which could explain the apparent immunosuppression observed in Group 2 mice from 48h onwards. Suppression of the immune system was also observed by Lammert and Wyrick (1982) in a model of peritoneal infection of mice with C. *psittaci*. In contrast to Group 2 mice, mice in Group 1 responded with significant levels of both macrophages and lymphocytes at 48h, indicating a progressive immune response against chlamydiae, possibly mediated by activated macrophages. This could also be responsible for the second influx of PMNLs observed at 5 days after infection in Group 1.

Interestingly, mast cell numbers only increased significantly in one instance; 5 days after infection in group 1 mice. Again, the lack of response in Group 2 mice may have been due to suppression of the immune system. In contrast to earlier hypotheses, the role of mast cells in clearance of Gram-negative bacteria may be considerable (Malaviya *et al.*, 1996) and indeed mast cells may modulate neutrophil influx at sites of infection through secretion of TNF-α. In the present study, mast cell accumulation was accompanied by a simultaneous increase in PMNL levels, and although cytokine levels were not determined here, other studies have demonstrated the secretion of TNF-α in response to chlamydiae (Williams *et al.*, 1989) and anti-chlamydial effects of TNF-α both *in vitro* (Shemer-Avni *et al.*, 1988) and *in vivo* (Williams *et al.*, 1990) have been observed.

Chlamydial antigen was rapidly detected by immunoperoxidase staining inside PMNLs at 3h after infection of mice with heat-killed chlamydiae (Group 3). No other groups were positive this early after inoculation and since staining did not occur subsequently in Group 3, this pattern of staining could represent ingestion and rapid degradation of non-viable chlamydiae.

In contrast, chlamydiae opsonised with immune serum were detected from 8h to 72h inside peritoneal cells. Initially, the main positive cell type was PMNLs however this

gradually changed to predominantly macrophages as the profile of cells in the peritoneal cavity altered. The reason for the detection of antigen at different times with heat-killed chlamydiae and organisms opsonised with immune serum, may be due to different processing pathways by cells of the immune system. Opsonised bacteria may invoke internalisation via FcγRIII receptors present on the surfaces of PMNLs and macrophages. This may result in a different degradation pathway to chlamydiae internalised by other means such as heat-killed chlamydiae.

Only chlamydiae pre-treated with naive serum were detected in a form which resembled the inclusions typically visible by Giemsa staining of cell cultures. These occurred after 48h and were restricted to the macrophage population. Larger areas of staining appeared with time leading to the conclusion that these did indeed represent developing chlamydiae.

Thus it would appear that neutralisation occurred in the peritoneal cavity of mice injected intraperitoneally with chlamydiae pre-opsonised with sheep immune serum and that organisms were internalised by both PMNLs and macrophages. Whereas chlamydiae pre-opsonised with naive serum gradually developed large granular inclusions inside macrophages, those incubated in the presence of immune serum resulted in progressively smaller foci indicating the non-viability of these organisms. The possibility that some chlamydiae persisted in a non-detectable form can not be ignored, however two events would argue against this. Firstly, even at 5 days after infection significant levels of immune cells were detected in the peritoneal cavity, indicating considerable stimulation of the immune system. Rasmussen *et al.,* (1996) recently showed evidence that aberrant or persistent forms of chlamydiae *in vitro* were not protected from cytotoxic attack. If this was also the case *in vivo*, then cells infected with persistent forms of chlamydiae would probably be killed. In addition, Beatty *et al.,* (1994) demonstrated immunochemical staining of persistently infected cells by MOMP-specific monoclonal antibodies, as long as fixative which caused permeation of the cell membrane was used. In the present study, such a fixative was employed as demonstrated by the internal staining of developing inclusions. Lack of

positive antigen detection was therefore probably not due to inability of reagents to permeate fixed cells, nor to a lack of specific antigen against which primary antibody solution could react. The most likely explanation for disappearance of visible chlamydial antigen was that chlamydiae pre-opsonised with immune serum (Group 1) were degraded in PMNLs and macrophages. In addition, only Group 2 mice were shown to increase chlamydial LPS levels in either the peritoneal cavity, liver or spleen throughout the experimental period.

# CHAPTER 6

## SERUM NEUTRALISATION ASSAYS *IN VITRO*

## 6.1. Introduction

Serum neutralisation assays (SNA) have been notoriously difficult to standardise for *Chlamydia* spp. (Byrne *et al.,* 1993), but their potential to identify protective antibodies, exemplified by other organisms such as influenza virus (Couch and Kasel, 1983) has maintained an interest in their development. Most chlamydial SNA are described for *C. trachomatis* and have been standardised to a high degree of consistency following an international workshop in 1993 (Byrne *et al.,* 1993). No such recognised methodology exists however, for *C. psittaci*. *In vitro* neutralisation with certain strains of *C. psittaci* have been described (Anderson *et al.,* 1988; Buzoni-Gatel *et al.,* 1990; Ando *et al.,* 1993), but these assays are not ubiquitous. This chapter describes a series of pilot experiments designed to develop an SNA for *C. psittaci*, abortifacient strain S26/3. The data represent preliminary results only and as such, no definitive conclusions could be drawn. During the development of the assay, several technical difficulties were encountered which impeded the development of the assay. The implications of these problems on future developments for a reliable SNA are discussed.

Neutralisation has been shown to occur *in vitro* either by interfering with the attachment phase of chlamydial particles (Ainsworth *et al.,* 1979), or post-attachment (Caldwell and Perry, 1982; Peeling *et al.,* 1988). Complement may (Howard,1975; Lucero and Kuo, 1985; Peterson *et al.,* 1988) or may not (Caldwell and Perry, 1982; Peeling *et al.,* 1988) be required. Furthermore, cells expressing different receptors, notably FcγIII, may affect the outcome of neutralisation (Su *et al.,* 1991). Some investigators have described differences in chlamydial binding to host cells by centrifugation-assisted infection (Ainsworth *et al.,* 1979; Caldwell and Perry, 1982). In addition, the composition of medium in which the assay is carried out may inhibit the neutralising action of antibodies (Peterson *et al.,* 1988). Each of these factors was investigated in this chapter using standard pools of immune and naive sheep sera. Monoclonal antibodies against *C. psittaci*, abortifacient strain S26/3, were also examined by SNA for protective capacity.

## 6.2. Materials and Methods.

### 6.2.1. Cell lines.

A panel of 6 cell types was examined for infectivity by *C. psittaci*, abortifacient strain, S26/3 (Table 6.1.). Inclusion bodies were counted on day 3 post-infection using a light microscope to visualise Giemsa positive cells. Monolayers of cells were produced on "Tracs" coverslips according to the methodologies detailed in Materials and Methods, section .

**Table** *6.1. Cell types examined for infectivity with* C. psittaci.

| | | |
|---|---|---|
| McCoy | - | mouse fibroblast cells |
| BHK-21 | - | Syrian hamster kidney cells |
| EBTr | - | bovine embryonic tracheal cells (fibroblastic) |
| L929 | - | mouse connective tissue (fibroblastic) |
| STC | - | sheep trophoblast cells (Morag Livingstone) |
| ST6 | - | sheep epithelial lymphoid cells (Dr M. Norval) |

### 6.2.2. Infection of cells.

#### 6.2.2.1. Materials and Methods.

*C. psittaci* inoculum was titrated in each cell line at 4 ten-fold dilutions (1 in 100 to 1 in 10,000). The cell lines was seeded at $2x10^5$ cells/ml on a glass coverslip in a sterile bijou (Trac) and allowed to monolayer overnight. BHK-21 cells were treated with IDU ( 80 μg/ml) 3 days prior to infection. All other cell types were treated with 1μg/ml of cycloheximide at the time of infection. The infection procedure for each cell line comprised the addition of 1ml of inoculum to each Trac, centrifugation at 2000x*g* for 30 min., followed by stationary incubation at 37°C/5% $CO_2$ for 3 days

with Trac lids loosened. Coverslips were removed from Trac bottles and stained with Giemsa (5% solution). Dark staining inclusion bodies were counted over the whole coverslip area. Mean values were obtained from duplicate coverslips.

#### 6.2.2.2. Results

Three cell types (EBTr, L929 and BHK-21) were prone to clumping which made identification and enumeration of inclusion bodies difficult (Table 6.1.). STC cells generally contained large inclusions, but the staining intensity of the inclusions was indistinct. These 4 cell types yielded comparable levels of infection. In contrast, McCoy cells were more susceptible to infection, with approximately 5-fold higher infection titres. In addition, although the inclusions were relatively small, they were easily recognisable and of uniform shape. ST6 cells which only became available late in the study, were extremely susceptible to infection, with multiple inclusions frequently present in a single cell. However, the inclusions were diffuse and identification of individual infected cells was problematic. Thus, McCoy cells were adopted as the standard cell type for further SNA studies.

### 6.2.3. Effect of centrifugation on infection.

#### 6.2.3.1. Materials and Methods.

The standard procedure was followed for infecting Tracs, except that after addition of inoculum to Tracs, half were centrifuged at 2000x*g* for 30 min., and half subjected to stationary incubation for the same period of time. A second experiment investigated the effects of different times of centrifugation up to 120 min., on the number of inclusions.

### 6.2.3.2. Results.

Centrifugation increased the number of inclusion bodies compared to stationary incubation (Table 6.3.a.) and this increase was related to the duration of centrifugation (Table 6.3.b.) (correlation coefficient, 0.78; $p>0.05$). The plot was sigmoid, with an initial sharp rate of increase in the number of inculsion bodies up to 30 min. centrifugation, falling off to a plateau with centrifugation times of 30-120 min.

## 6.2.4. Effect of adding divalent cations ($Mg^{2+}$) to infection medium.

### 6.2.4.1. Materials and Methods.

It has been stated (Peterson *et al.*, 1988) that divalent cations in the infection medium can have a detrimental effect on neutralisation *in vitro* and the extent of this effect is related to the concentration of divalent cations present. Sera which exhibited neutralising capacity under conditions of low divalent cation concentration, became non-neutralising as the concentration of these particular components increased. The effect of divalent cations on infection of McCoy cell monolayers was investigated by adding 800μM magnesium sulphate to the inoculum mixtures in duplicate Tracs

*Table 6.2.* **Infection of cell monolayers.**

| cell type | inoculum titre | Appearance of cells and inclusion bodies |
|---|---|---|
| McCoy | $1 \times 10^5$ | inclusion bodies small, round and distinct |
| BHK-21 | $5 \times 10^4$ | cells tended to clump; inclusions large and irregularly shaped |
| EBTr | $6 \times 10^4$ | cells clumped, inclusions difficult to identify |
| L929 | $3 \times 10^4$ | cells tended to clump; inclusions distinct |
| STC | $6 \times 10^4$ | large inclusions, but less distinct than in McCoys |
| ST6 | ND | multiple, but diffuse and indistinct inclusions |

ND, not determined.

- Chlamydial inoculum titrated at ten-fold dilutions over the range 1 in 100 to 1 in 100,000.
- Cells seeded at $2x10^5$ cells/ml and allowed to monolayer on glass coverslips.
- Monolayers infected with 1ml of diluted inoculum, centrifuged at 2000x*g* for 30 min.
- After incubation of infected cells for 3d at 37°C/5% $CO_2$, cells were stained with 5% Giemsa solution and inclusion bodies counted over the whole coverslip.

***Table 6.3.*** *a) Effect of centrifugation on infection of cells.*

| **Inoculum dilution** | **inclusions per coverslip** | |
|---|---|---|
| | **centrifuged** | **Stationary** |
| $1 \times 10^{-1}$ | > 500 | > 500 |
| $1 \times 10^{-2}$ | > 500 | 204 |
| $1 \times 10^{-3}$ | 424 | 26 |
| $1 \times 10^{-4}$ | 30 | 2 |
| $1 \times 10^{-5}$ | 0 | 0 |

b) *Effect of increasing centrifugation time on infection.*

| **Inoculum** | **centrifugation time (min.)** | **inclusions (ifu/ml)** |
|---|---|---|
| S26/3, $7^{th}$ passage | 0 | 16 |
| | 10 | 427 |
| | 30 | 676 |
| | 60 | 751 |
| | 120 | 810 |

- Cells seeded at $2x10^5$ cells/ml and allowed to monolayer on glass coverslips.
- Monolayers infected with 1ml of diluted inoculum, a) centrifuged at 2000x*g* for 30 min. or left at room temperature for equivalent time, b) centrifuged as stated or incubated immediately as below.
- After incubation of infected cells for 3d at 37°C/5% $CO_2$, cells were stained with 5% Giemsa solution and inclusion bodies counted over the whole coverslip.

### 6.2.4.2. Results.

Table 6.4. shows the effect of adding magnesium sulphate (800μM), on the infectivity of *C. psittaci*. This addition increased the infectivity of the chlamydial inoculum. The mechanism of the increase remained unclear, but since this effect might also potentially abrogate neutralisation, as with *C. trachomatis* (Peterson *et al.*, 1993), levels of $Mg^{2+}$ cations were minimalised in future experiments by using RPMI as diluent for inoculum mixtures and as culture medium as far as possible.

***Table 6.4.*** *Effect of divalent cations on infection of cells.*

| **Addition of $MgSO_4$** | **Inclusions (ifu/ml)** |
|---|---|
| 0 | 676 |
| 800 μM | 1094 |

- Refer to Table 6.3. for legend
- Magnesium sulphate was added at a concentration of 800μM where stated
- Monolayers were centrifuged for 30 min. at 2000x*g*.

## 6.3. Neutralisation of infection.

### 6.3.1. Materials and Methods.

Immune sera from ewes which had aborted following experimental infection with *C. psittaci* and a serum pool from ewes naive to abortifacient *C. psittaci* were tested at a concentration of 25% in the infection medium used above. Inoculum mixtures were prepared by diluting the chlamydial suspension to a working concentration of $1x10^3$ ifu/ml in RPMI. Heat-inactivated sheep sera were also diluted 1 to 1 in RPMI. Both constituents were warmed to 37°C before being mixed in a 1:1 ratio which included, where stated, complement in the form of guinea pig serum (Sigma Ltd) at 5% final volume. The mixtures were vortexed and incubated at 37°C for 45 min. in a water bath, with occasional mixing. After 45 min., inoculum mixtures were added in 1ml volumes to cell monolayers in Tracs in duplicate. The Tracs were centrifuged at 2000x*g* for 60 min. followed by 2h stationary incubation at 37°C in 5% $CO_2$. Tracs were then rinsed with 3 changes of pre-warmed PBS (37°C) and 1ml of cell maintenance medium added to each Trac (2% serum content). The cells were incubated for 3d at 37°C/5% $CO_2$ then stained with 5% Giemsa solution and inclusion bodies counted under a light microscope (x200 magnification). The procedure was performed twice with pooled serum samples (Experiments A and B, Table 6.5) and again using individual sera which had constituted the pool of immune serum. The result was to generate a range of neutralising capacities representative of a population of sheep experimentally infected with *C. psittaci*.

### 6.3.2. Results.

The mean number of inclusion bodies counted over 5 sample fields of view (x 200 magnification) in duplicate Tracs were calculated. Neutralisation was estimated as the

percentage reduction in inclusion bodies with test serum samples compared with the naive serum control:-

*% Neutralisation = 100 - (immune serum/naive serum x 100)*

The range of neutralisation in a sample of 8 immune sera are shown in Table 6.6. Two modes of neutralisation were apparent, one dependent on the presence of intact complement (GPS) and the other, not. These contributed approximately 15% and 75% respectively to overall neutralisation (sem 0.7 and 3.3 respectively). The majority of neutralisation therefore appeared to be due to a complement-independent mechanism in this particular system.

Further experiments using immune and naive sera gave variable results and in some cases, no neutralisation was evident with the standard immune serum. Several experiments resulted in so few inclusion bodies that calculation of neutralisation was impossible. The reason for these discrepancies was unclear, since all parameters including inoculum batch were standardised.

One batch of inoculum produced consistently high neutralisation levels (86-100%) with standard immune serum pool and only fell below this level when serum contents of the inoculum mixture was reduced to 2.5% or less (Table 6.6). Serum dilutions of 1/2-1/10 (final volume) produced equivalent amounts of neutralisation ($p>0.05$). In these conditions, the effect of exogenous complement was minimal, since all assays were performed in the absence of complement.

## 6.4. Neutralisation by Monoclonal antibodies.

### 6.4.1. Materials and Methods.

A range of monoclonal antibodies (MAbs) was assessed by SNA. These included MAb 4/11, a MOMP-specific MAb against variable segment 2 (IgG2b isotype), MAb 4/40 also against MOMP, but not VS2 (IgG2a isotype) and MAbs 13/4 and 13/5, against the genus-specific LPS epitope of *C. psittaci* (IgG3 isotype). All MAbs were used as heat inactivated, unpurified ascitic fluids at 1/100 dilution. Exogenous complement was not included. Naive sheep serum was again used as the control.

### 6.4.2. Results.

The results in Table 6.7. show that 70-88% neutralisation was achieved with the genus-specific MAb 13/4 and 13/5. In contrast MAbs 4/40 and 4/11 induced only 26% and 34% neutralisation, respectively.

***Table 6.5.*** *Neutralisation* in vitro *by pool of immune sheep serum.*

| | **Number of inclusion bodies per 5 fields of view (x200 magnification)** | | | | | | **% neutralisation** *100 - [immune x 100] [ naive]* | | |
|---|---|---|---|---|---|---|---|---|---|
| **serum** | **Immune serum** | | | **naive serum** | | | | | |
| **dilution** | **Expt. A** | **Expt. B** | **Expt. C** | **Expt. A** | **Expt. B** | **Expt. C** | **Expt. A** | **Expt. B** | **Expt. C** |
| **1/2** | 9 | 12 | 32 | 166 | 277 | 274 | **95** | **96** | **88** |
| **1/4** | 8 | 21 | 34 | 174 | 434 | 374 | **95** | **95** | **91** |
| **1/10** | 7 | 25 | 29 | 149 | 450 | 498 | **95** | **94** | **94** |
| **1/20** | 9 | 25 | 37 | 175 | 384 | 414 | **95** | **94** | **91** |

- *% Neutralisation = 100 - (immune serum/naive serum x 100)*

- Refer to Table 6.4. for legend.

***Table 6.6.*** *Neutralisation* in vitro *by individual serum samples from immune sheep.*

| Immune Serum (post-OEA) | Inclusions per 5 Fields of View (x200 magnification) | | % Neutralisation | |
|---|---|---|---|---|
| | **+ GPS** | **H.I. GPS** | **+ GPS** | **H.I. GPS** |
| 1 | 40 | 86 | 82 | 63 |
| 2 | 22 | 16 | 90 | 93 |
| 3 | 36 | 80 | 83 | 66 |
| 4 | 22 | 50 | 90 | 78 |
| 5 | 8 | 56 | 96 | 76 |
| 6 | 22 | 50 | 90 | 78 |
| 7 | 4 | 48 | 98 | 79 |
| 8 | 16 | 72 | 93 | 69 |
| ***mean±sem*** | - | - | ***90 ± 0.7*** | ***75 ± 3.3*** |

**H.I.** - Heat inactivated.

Refer to Table 6.4. for legend, except that heat inactivated serum was incorporated at 1/4 final dilution.

- Neutralisation was calculated as ***100 - [immune/ naive x 100]***, where naive serum with and without GPS resulted in 218 and 232 inclusions per 5 fields of view (x200 magnification), respectively.

*6.7. Neutralisation* in vitro *by monoclonal antibodies.*

| Antibody | specificity | isotype | [1]% neutralisation |
|---|---|---|---|
| MAb 4/11 | MOMP (VS2) | IgG2b | 34 ± 7.5 |
| MAb 4/40 | MOMP | IgG2a | 26 ± - |
| MAb 13/4 | LPS | - | 70 ± 3.5 |
| MAb 13/5 | LPS | IgG3 | 88 ± 0.5 |
| [2]immune sera | polyclonal | IgG1/2 | 100 ± 0 |

[1] mean of duplicate infected Tracs ± sem.

[2] standard pool of sheep immune sera.

Refer to Table 6.4. for legend, except that heat inactivated MAbs were used at 1/100 final dilution.

## 6.4. Discussion

Initially it proved exceedingly difficult to demonstrate consistent *in vitro* neutralisation of chlamydial infectivity. Infection was standardised by using confluent monolayers of McCoy cells on 16mm diameter coverslips and infecting with $1x10^3$ ifu of *C. psittaci*, strain S26/3. Infection was assisted by centrifugation, otherwise an insufficient number of inclusions were formed. This was possibly a reflection of the predilection of the ovine *C. psittaci* for macrophages and trophoblast cells. Indeed, although the titre was no higher in trophoblasts than in McCoy cells, inclusion bodies were considerably larger in the former cell type. Caldwell and Perry (1982) observed that centrifugation may result in an alternative mechanism of internalisation to stationary incubation, although it is unknown which mode of uptake more commonly occurs in natural infections. Several other groups have also postulated various mechanisms for chlamydial uptake including pinocytosis (Reynolds and Pearce, 1991) and phagocytosis (Prain and Pearce, 1989), each mode dependent on either static or centrifugal conditions respectively, at the time of infection. Although chlamydial entry into host cells appears deeply complex, it is not, as Reynolds and Pearce (1991) point out, an isolated occurrence. Other cases in which multiple endocytic pathways are utilised have been documented for intracellular bacteria such as *Salmonella spp.* (Elsinghorst *et al.*, 1990; Finlay and Falkow, 1989) and for *Yersinia spp.* (Isberg, 1989).

Interestingly, Prain and Pearce (1989) highlighted a difference in productive infection between centrifuged and stationary infected cells, which appeared to result from post-entry events associated with selection of the surrounding vacuole membrane such that centrifuged chlamydiae utilised a phagocytic mechanism during internalisation, subsequently adopting an alternative vacuole membrane to that with pinocytic entry and consequently avoiding phagolysosomal-fusion. M$^c$Clarty (1994) also alluded to a similar occurrence, suggesting that chlamydiae may utilise a mechanism of internalisation normally associated with uptake of nutrients into host cells whereupon not all ingested

vacuoles are ultimately destined for degradation and may consequently avoid fusion with lysosomal compartments. Again the question arises as to whether establishment of chlamydial infection under manipulated conditions *in vitro* allows accurate assessment of methods for preventing infection occurring.

In one instance, an elevated concentration of divalent cations in the infection medium combined with centrifugation, resulted in increased levels of infection. The reason for this is unclear, however it may simply have been a reflection of optimising magnesium ion levels in the infection medium with regards their necessity for chlamydial ATPase activity (Peeling *et al.,* 1989). In addition, magnesium ions appear to cross-link the LPS moiety of the outer membrane of chlamydiae (Vretou *et al.,* 1992) and probably affect exposure of epitopes situated on the MOMP. This may be beneficial to chlamydiae under physiological conditions by hindering access of antibodies to neutralising epitopes. Peterson *et al.* (1988) found this to be the case *in vitro* when neutralisation by monoclonal antibodies was abrogated by high levels of magnesium ions.

In the series of experiments reported here, individual sera from immune sheep reduced infection of McCoy cells with chlamydiae by between 82-98% when an exogenous source of complement was added. If complement was omitted, neutralisation fell to between 63-93%. Thus, although complement enhanced neutralisation of infection under these conditions, the majority of infectious particles appeared to be neutralised by a complement-independent mechanism. Since internalisation of chlamydiae is thought to be directed by the bacterium rather than through activity of the host cell (Byrne and Moulder, 1978), these results suggest that complement may enhance the association between chlamydial and host cell membranes thereby aiding internalisation. Furthermore, it has been shown (Hall *et al.,* 1993) that MOMP was the primary binding site for activated complement, and although activation was most effective by the antibody-independent alternative pathway, this further implicates MOMP as an important structure in the uptake of chlamydiae. Previous data (Eissenberg *et al.*, 1983)

had highlighted the absolute requirement of elementary body envelopes in ingestion of chlamydiae.

The findings with monoclonal antibodies in the SNA were anomalous. The monoclonal antibodies specific for MOMP (4/11 and 4/40), including one specific for VS2 (4/11), yielded only low (34%) levels of neutralisation. This is in contrast to the *in vivo* findings reported in subsequent chapters and documented for *C. trachomatis*. In contrast, anti-LPS antibodies are generally considered not to be neutralising (Salinas *et al.*, 1994). However, the two anti-LPS MAbs evaluated (13/4 and 13/5) induced 70-80% neutralisation in the present study. The lack of complement in the inoculation mixtures may have caused the low neutralisation results found with MAbs 4/11 and 4/40, although the immune sheep serum used induced 100% neutralisation. Furthermore, VS2 is thought to contain at least one protective B-cell epitope (Batteiger, 1996) and would have been expected to have caused neutralisation in the present study. Therefore, it is conceivable that conditions under which neutralisation assays were performed were not favourable and possibly encouraged interaction of magnesium ions with chlamydial LPS thereby abolishing neutralisation by monoclonal antibodies specific to MOMP. Conversely, the presence of high levels of anti-LPS antibodies either in the form of monoclonals or in immune sheep sera may have obstructed binding of magnesium ions to chlamydiae by binding themselves to LPS epitopes. Overall, these results suggest that the modes of neutralisation *in vivo* and *in vitro* are not entirely comparable at present and can be significantly affected by modification of the infection process in vitro.

# CHAPTER 7

## ASSESSMENT OF PROTECTION OF NOVEL RECOMBINANT ANTIGENS OF MOMP

## 7.1 Introduction

Immunisation of sheep with tissue-grown, inactivated chlamydial antigen successfully protected sheep against chlamydial abortion (Jones *et al.,* 1994). However, the method of production was highly demanding technically and a more commercially viable vaccine was sought. Previously, it had been possible to demonstrate high levels of protection in sheep using a semi-purified chlamydial outer membrane complex (COMC) preparation (Tan *et al.,* 1990). It was therefore envisaged that if the MOMP molecule was reproduced on a large scale by recombinant protein technology, similar protection could be expected.

Neutralising antibodies against *Chlamydia* have been identified and shown to correspond to epitopes in VS1, 2 and 4, of MOMP. In particular, VS1 appears to be highly immunogenic and with its charge and surface exposure, may induce a protective response. Work by Villeneuve *et al.,* (1994) indicated that immunogenicity of MOMP may be determined by the surface availabilities of MOMP VSs and not their primary sequences. Taking this into account, it was important to generate recombinant MOMP molecules which more importantly than having identical amino acid sequences to native MOMP, actually folded into a three-dimensional structure which exposed the same antigenic regions as the original.

Experiments designed to elucidate the protective immune response against *C. psittaci*, showed that both CMI and humoral immunity played a role in protection. Transfer of primed T-cells and of immune sera in mice conferred resistance to infection (Buzoni-Gatel, Rodolakis and Plommet, 1987). Likewise, immunity to reinfection of guinea pigs with *C. psittaci* (GPIC) appeared to be dependent on an intact humoral immune response (Rank and Barron, 1983). The immunogenicity efficacies of recombinant antigens were examined in mice and compared to the results in sheep.

## 7.2. Design of MOMP Constructs

Recombinant MOMP constructs were kindly gifted by Dr Alan Herring (Department of Biochemistry, Moredun Research Institute). A summary of the expression of recombinant constructs of MOMP in standard *E. coli* systems is given in Chapter 2. The table below summarises the MOMP constructs included in the present study.

| Acronym | Name | Site |
|---|---|---|
| fMOMP-pD | fusion MOMP | insoluble inclusions in cytoplasmic space |
| mMOMP | membrane MOMP | detergent extraction in outer membrane |
| mMOMP19 | membrane MOMP | probably in periplasmic space |
| tMOMP | truncated MOMP | insoluble inclusions in cytoplasmic space |

## 7.3. Materials and Methods.

Recombinant antigens described above were analyzed for immunogenicity and efficacy using the following techniques:-

a) immunoblotting and indirect ELISA
b) stimulation of peripheral blood leukocytes *in vitro*
c) *in vitro* serum neutralisation
d) protection in mice (i) active immunisation - Model 1, (ii) passive immunisation - Model 2.

The results were compared to the performances of the recombinant antigens in challenge experiments involving pregnant ewes, which were carried out by the Department of Chlamydiology and involving myself as part of a larger team.

**7.3.1. Protective efficacies in pregnant sheep:** The protective efficacies of recombinant vaccines against *C. psittaci* were examined in pregnant sheep. No universally accepted method of categorising sheep as protected or otherwise has been agreed and so several criteria were assessed by Dr. G. E. Jones with respect to the ewe and to pregnancy. These were:- a) number of dead lambs/abortions, b) mean gestation period, c) % placental lesions, d) proportion of placental area grossly affected and e) % of placentas positive for *C. psittaci* by modified Ziehl-Neelson staining/culture/LPS-ELISA. A final combined score incorporating each of these parameters was calculated for each group and expressed as a protective index (PI%) (Table 7) (Jones *et al.*, 1993).

**7.3.2. Immunoblotting and indirect ELISA of sera:** Sera from sheep vaccinated with recombinant antigens were pooled from groups of between 3-4 animals and tested by indirect ELISA (I. E. Anderson) and immunoblotting against native chlamydial antigen. Pools of sera were used rather than individual samples, since it would be impossible to test every sample in mice with sufficient group sizes to be statistically significant. Therefore, it was deemed reasonable to assess ovine immune responses to vaccination on a group basis for each assay performed.

Briefly, the indirect ELISA was performed by coating ELISA plates with soluble chlamydial antigen overnight at 4°C (Anderson *et al.*, 1994). The coating antigen was prepared by I. E. Anderson by sarkosyl extraction from whole chlamydial elementary bodies. Plates were treated with periodate, blocked with 10% horse serum and fixed with glutaraldehyde solution. Sera were tested at 1/800 dilution and chlamydia-specific antibodies detected using donkey anti-sheep IgG specific antibody conjugated to HRP. The substrate used was OPD, stopped at an appropriate time with 0.2M sulphuric acid. Immunoblots were also performed using heat-treated, SDS-solubilised chlamydial antigen.

**7.3.3. Stimulation of IFN-γ *in vitro*:** Two recombinant forms of MOMP available at the start of the project (fMOMP and mMOMP) were used to vaccinate groups of 7

sheep on 2 occasions, 3 weeks apart. Each vaccination contained approximately 50μg/ml protein and volumes of 1ml were administered subcutaneously on each occasion. Venous blood was taken from each animal approximately 21 days after second vaccination and collected into evacuated tubes containing lithium heparin (no preservative) at a final concentration of 15 units/ml. Aliquots of 1.5ml blood were mixed with 100 μl of appropriate antigen and the mixtures incubated overnight at 37°C.

The following day, mixtures were centrifuged and the upper layer comprising PBLs collected. Reactivity of PBLs was tested *in vitro* using interferon-gamma (IFN-γ) production as a measure of the reactivity index of PBLs (Central Science Laboratories, Bovine γ- interferon kit). Two forms of native chlamydial antigen were used to detect PBL sensitisation *in vitro*; semi-purified chlamydial elementary bodies and a solubilised, purified MOMP preparation. Non-specific reactivity of PBLs was measured by inclusion of an LPS control in the form of a B-cell mitogen (*Salmonella minnesota Re* mutant; Sigma Ltd) and a T-cell mitogen (concanavalin A).

**7.3.4. Serum neutralisation assays:** The serum neutralisation assay (SNA) (Chapter 6) was applied to sera from sheep vaccinated with fMOMP and tMOMP. Immune and naive controls were formed by the serum pools described previously (Chapter 6) diluted 1/2, 1/4, 1/10, 1/20, 1/40 and 1/80. Test serum samples were also diluted over this range.

**7.3.5. Active immunisation-Model 1:** The recombinant proteins were examined for protective capacity using the model of active immunisation in mice (Chapter 4). In the first experiment, mMOMP and tMOMP were tested and in the second experiment, mMOMP19 and tMOMP. Briefly, mice were immunised subcutaneously with 5μg antigen on 2 occasions, 3 weeks apart. Three weeks after the second vaccination, mice were challenged intraperitoneally with $1x10^6$ ifu and killed 6 days later. Liver and spleen tissues were analysed indirectly for chlamydial infection using a sandwich ELISA to detect LPS.

**7.3.6. Passive immunisation - Model 2:** Two phases were necessary to complete passive immunisation in mice. Initially, sheep were immunised using the test vaccines, followed by a second phase in which sera from the vaccinated sheep were incubated with live chlamydiae and injected into mice.

**Phase 1; Vaccination of lambs with recombinant proteins:** Groups (n=7) of 6 month old lambs were immunised subcutaneously with 50μg of recombinant protein per animal on 2 occasions, 3 weeks apart and blood samples taken 3 weeks after the second vaccination (day 42). The recombinant MOMP forms used were mMOMP and tMOMP, prepared as both soluble and insoluble forms. Pools were constituted for each group using an equal volume of serum from each group member. MOMP-specific antibody levels were quantified for individual animals using the indirect ELISA described previously (section 7.3.2.).

**Phase 2; Injection of mice:** Equal volumes of pooled serum (25% final volume) and chlamydial inoculum (1-2x$10^6$ ifu/ml, final titre) were combined to form inoculation mixtures. After incubation at 37°C for 45 mins, 1ml of mixture was injected intraperitoneally into each mouse and the mice killed 6 days later. Liver and spleen tissue were analysed for chlamydial infection by LPS ELISA.

## 7.4 Results.

### 7.4.1 Protective efficacies in pregnant sheep

Data in this section were collected by myself and members of the Chlamydiology section over a 4 year period, in collaboration with Hoechst Animal Health Ltd. These results are presented in order to determine any correlations between the *in vitro* techniques and mouse models applied, compared with the efficacies observed with the recombinant MOMP vaccines in their natural host and disease, namely pregnant ewes and OEA.

*Table 7.1. Design of experiment 1: Assessment of protective efficacies of recombinant proteins of* C. psittaci *MOMP.*

| Group | n | vaccine/serum source |
|---|---|---|
| 1 | 10 | mMOMP; lambs |
| 2 | 10 | soluble mMOMP; lambs |
| 3 | 10 | tMOMP; lambs |
| 4 | 10 | soluble tMOMP; lambs |
| 5 | 10 | native chlamydial antigen; lambs |
| 6 | 20 | immune serum; convalescent ewes |
| 7 | 10 | naive serum; adult ewes |

**7.4.1.1 Immunoblotting and indirect ELISA analysis of sheep sera:** Pools of sera from sheep vaccinated with recombinant antigens gave strong positive responses by both indirect ELISA and immunoblotting in all cases (Table 7.3., Fig. 7.1., respectively).

***Legend for Table 7.2.*** *Protection in pregnant ewes against challenge with* C. psittaci.

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- ewes vaccinated with 2x50μg of recombinant antigen, followed by s.c. challenge at 70-73 days gestation with $5x10^5$ ifu *C. psittaci* (S26/3 + S95/3)
- abortion defined as premature delivery of dead lambs or of lambs dying within 48h due to no discernible cause other than chlamydial infection; mean number of dead lambs and length of gestation calculated per group; placental material analysed for chlamydial infection by culture/modified Ziehl-Neelson staining/ELISA detection of LPS
- Combined protection score calculated from individual parameters:- Sum of [dead lamb ratio x 0.5] + [% ewes aborting x 0.3] + [% ewes +ve for *C. psittaci* x 0.2]

$$PI\,\% = 100 \times \left[1 - \frac{[\text{Test value} - \text{Negative control value}]}{[\text{Positive control value} - \text{Negative control value}]}\right]$$

---

*Table 7.2. Protection in pregnant ewes against challenge with* C. psittaci.

| group | treatment | n | Mean gestation period (days) | % of ewes aborting | % ewes positive | Dead lamb ratio | Combined score (PI%) |
|---|---|---|---|---|---|---|---|
| 1 | fMOMP | 22 | 137.4 ± 1.9 | 41 | 68 | 37 | 44 (24%) |
| 2 | not vaccinated, infected | 24 | 136.2 ± 1.7 | 50 | 83 | 53 | 58 |
| 3 | not vaccinated, not infected | 21 | 144.8 ± 0.3 | 0 | 0 | 0 | 0 |
| 1 | 100µg mMOMP | 22 | 137.2 ± 1.9 | 46 | 73 | 48 | 52 (0%) |
| 2 | not vaccinated, infected | 23 | 140.2 ± 1.5 | 35 | 70 | 33 | 41 |
| 3 | not vaccinated, not infected | 21 | 146.2 ± 0.6 | 0 | 0 | 0 | 0 |
| 1 | tMOMP soluble | 13 | 141.5 ± 1.9 | 31 | 46 | 29 | 33 (65%) |
| 2 | tMOMP insoluble | 14 | 141.8 ± 2.0 | 21 | 50 | 17 | 25 (54%) |
| 3 | mMOMP 19 | 16 | 135.1 ± 2.7 | 44 | 75 | 50 | 53 (26%) |
| 4 | not vaccinated, infected | 23 | 134.7 ± 1.8 | 65 | 87 | 69 | 71 |
| 5 | not vaccinated, not infected | 12 | 144.6 ± 0.4 | 0 | 0 | 0 | 0 |

See section 7.4.1.1 for legend.

***Table 7.3.*** *Seroconversion in response to vaccination of sheep with recombinant antigens.*

| group | vaccine antigen/adjuvant | ELISA $OD_{492}$ (nm) | |
|---|---|---|---|
| | | 21 dpv | 42 dpv |
| 1 | fMOMP 1, M/A | 1.387 | 1.628 |
| 2 | fMOMP 1, B/W | 0.762 | 0.808 |
| 3 | fMOMP 2, M/A | 1.488 | 1.667 |
| 4 | soluble mMOMP, M/A | 0.936 | 1.379 |
| 5 | EBs, M/A | 1.708 | 1.790 |
| 6 | EBs, B/W | 0.587 | 1.114 |
| 7 | purified native MOMP, M/A | 1.620 | 1.812 |
| 8 | placebo, M/A | 0.301 | 0.344 |
| 9 | placebo, B/W | 0.273 | 0.296 |

**Key: M/A,** Marcol/Arlacel A + Alhydrogel; **B/W,** Beringwerke W19

- Groups of sheep (n=7) were vaccinated on 2 occasions, 3 weeks apart with adjuvanted recombinant protein. A total antigen payload of 100μg was injected into each animal.

- Blood samples taken on days 21 and 42 post-primary vaccination were assayed for antibodies in an indirect ELISA using soluble chlamydial antigen.

Key: 1, fMOMP 1, M/A

2, fMOMP 1, B/W

3, fMOMP 2, M/A

4, soluble mMOMP, M/A

5, EBs, M/A

6, EBs, B/W

7, purified native MOMP, M/A

8, placebo, M/A

9, placebo, B/W

10, +ve control (convalescent ewes)

11, -ve control (naïve lambs).

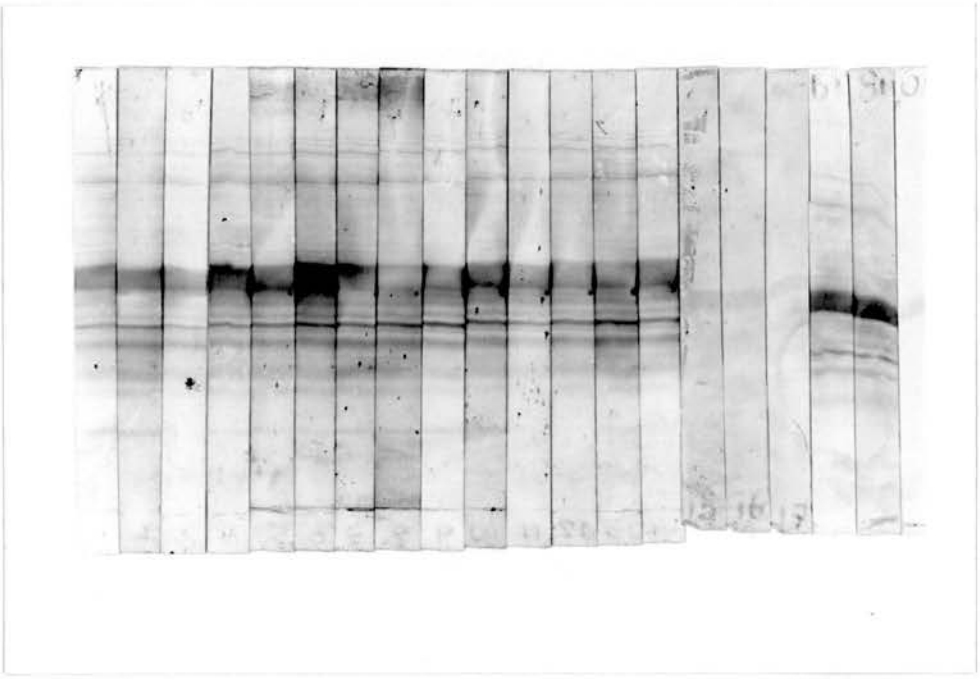

**Fig. 7.1.** Immunoblot of sera from ewes vaccinated with recombinant antigens.

**7.4.1.2 Reactivation of PBLs *in vitro*:** No significant production ($p>0.05$) of IFN-γ was observed when PBLs from placebo vaccinated sheep were incubated with *C. psittaci*, strain S26/3 (Fig 7.2.). In contrast, incubation of PBLs from sheep vaccinated with native chlamydial antigen resulted in significant release of IFN-γ ($p<0.01$), indicating recognition of chlamydial epitopes by sensitised PBLs. Similarly, PBLs from ewes which had aborted due to *C. psittaci*, induced significant production of IFN-γ ($p<0.05$) on incubation with native chlamydial antigen. The differences in IFN-γ levels produced by antigen concentrations ranging between 5μg/ml-40μg/ml were not significant ($p>0.05$), therefore further assays using recombinant antigen vaccines were only tested with 5μg/ml of chlamydial antigen *in vitro*.

Results using PBLs from sheep vaccinated with recombinant antigens (Table 7.4a) indicated that both forms of fMOMP were sufficiently similar to the native form of MOMP in chlamydial EBs to stimulate significant IFN-γ production *in vitro* ($p<0.05$). The same was true with solubilised, purified native MOMP ($p<0.01$) (Table 7.4b). In contrast, PBLs from sheep vaccinated with soluble mMOMP were unable to produce significant levels of IFN-γ ($p>0.05$), unless the antigen used *in vitro* was solubilised, purified native MOMP ($p<0.01$).

***Legend for Fig 7.2*** *Stimulation of PBLs in vitro.*

- *Lambs were vaccinated with 2x50μg of antigen or placebo (n=7).*
- *Unclotted blood samples were collected in heparin tubes and buffy coats removed for analysis.*
- *Samples were incubated with intact chlamydial elementary bodies and production of interferon-gamma measured by ELISA. Increases in IFN-γ levels were indicative of recognition of the stimulatory antigen by PBLs.*

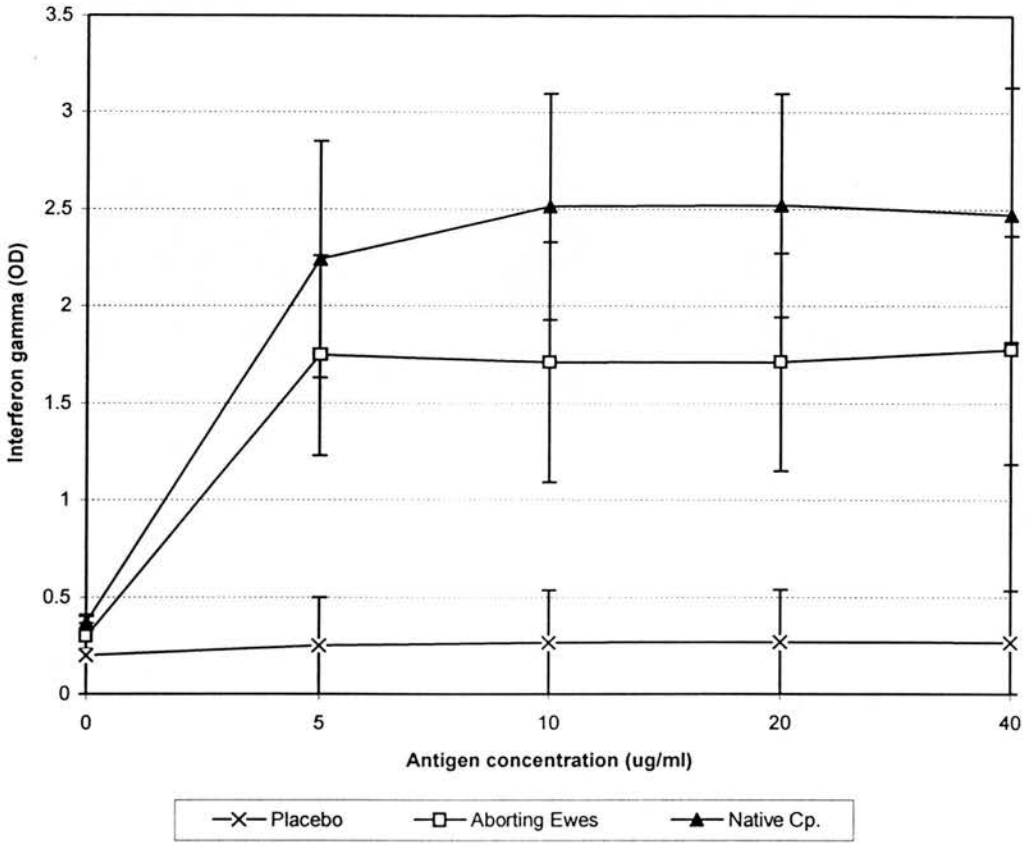


***Fig 7.2.*** *Reactivation of PBLs* in vitro; IFN-γ production by PBLs from placebo vaccinated sheep when incubated *in vitro* with whole *C. psittaci* EBs.

*Production of IFN-γ was significantly different when antigen was present, compared to no antigen using PBLs from sheep vaccinated with native* C. psittaci *($p<0.05$) or from aborted ewes ($p<0.01$).*

*Refer to section. 7.4.1.2 for legend.*

***Tables 7.4.*** *Reactivation of PBLs* in vitro.

a) IFN-γ production by PBLs from sheep vaccinated with recombinant antigens when incubated *in vitro* with whole *C. psittaci* EBs.

| vaccine | n | IFN-γ release ($OD_{450}$) | |
|---|---|---|---|
| | | **mean** | *sem* |
| fMOMP 1 | 7 | **0.449*** | *0.15* |
| fMOMP 2 | 7 | **0.439*** | *0.19* |
| soluble mMOMP | 7 | **0.515** | *0.27* |
| whole EBs | 7 | **0.893**** | *0.28* |
| purified native MOMP | 7 | **1.560**** | *0.17* |
| placebo | 4 | **0.070** | *0.04* |

b) IFN-γ production by PBLs from sheep vaccinated with recombinant vaccines when incubated *in vitro* with solubilised, purified MOMP.

| vaccine | n | IFN-γ release ($OD_{450}$) | |
|---|---|---|---|
| | | **mean** | *sem* |
| fMOMP 1 | 7 | **1.056**** | *0.22* |
| soluble mMOMP | 7 | **1.214**** | *0.45* |
| purified native MOMP | 7 | **2.334**** | *0.34* |
| placebo | 4 | **0.046** | *0.03* |

***Key:*** *Significantly higher than placebo; * $p<0.05$, ** $p<0.01$ (Student's t-test).*

*Refer to Fig 7.2 for legend, except that results in Table 7.4b represent samples incubated with solubilised, purified MOMP.*

**7.4.1.3 Serum neutralisation assays (SNA):** In Chapter 6, validation of the SNA was problematic, however considering these difficulties the following preliminary results were obtained using sera from sheep vaccinated with recombinant antigens. Immune serum resulted in a large reduction in the number of inclusion bodies (> 85%) compared with naive serum, indicating that substantial neutralisation of chlamydial infection was occurring with this assay. Neutralisation by immune sheep sera diluted to 1/2 and 1/40 was considerable, although because only duplicates were used, no statistical significance could be attributed to the result. At the highest dilution (1/40), approximately 89% neutralisation was obtained, with levels decreasing only when immune sera was diluted beyond 1/80. A similar trend was seen with sera from sheep vaccinated with tMOMP. Sera where divided into two groups depending on antibody titre. As a result, the pool of low titre sera produced considerably less neutralisation than the high titre pool at dilutions of 1/2 and 1/20. The neutralising capacities of the two serum pools were indistinguishable at all other dilutions. In contrast, sera from sheep vaccinated with fMOMP2 produced considerably lower neutralisation than the immune serum at dilutions of 1/2 to 1/20.

***Table 7.5.*** In vitro *neutralisation by recombinant antigens.*

| | % neutralisation (duplicates, A/B) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| serum dilution | fMOMP | | tMOMP (low titre) | | tMOMP (high titre) | | immune serum | |
| | A | B | A | B | A | B | A | B |
| 1/2 | 61 | 53 | 87 | 82 | 93 | 95 | 86 | 91 |
| 1/4 | 54 | 64 | 88 | 89 | 94 | 91 | 92 | 89 |
| 1/10 | 76 | 58 | 92 | 86 | 95 | 85 | 95 | 88 |
| 1/20 | 81 | 68 | 89 | 85 | 93 | 90 | 91 | 90 |
| 1/40 | 83 | 77 | 86 | 84 | 90 | 71 | 87 | 85 |
| 1/80 | 76 | 56 | 80 | 41 | 80 | 59 | 76 | 50 |

- *sheep vaccinated with 2x50μg doses of recombinant antigen,*
- *serum samples pooled for each group and mixed with an equal volume of chlamydial inoculum ($1x10^3$ ifu/ml): The SNA was performed as described in Table 6.4.*
- *Duplicate "Tracs" were counted for each dilution (A and B)*
- *% neutralisation = 100 - ( inclusion bodies with test sample x100) / (inclusion bodies with naive serum)*

### 7.4.2. Protective efficacies in mice.

**7.4.2.1. Active immunisation - Model 1:** Groups of 4 week old mice were vaccinated subcutaneously with 0.1ml of recombinant or native chlamydial antigen adjuvanted in Montanide ISA 50 with Alhydrogel. Recombinant antigens mMOMP, tMOMP and mMOMP19 were included in the vaccines at a concentration of 100 μg/ml, 100 μg/ml and 17 μg/ml, respectively. The vaccine prepared from VS1 and VS3 contained a final protein concentration of 32 μg/ml, made up of equal concentrations of each peptide. Native chlamydial vaccine comprised approximately 100 μgMOMP/ml (500 μg/ml of total chlamydial protein), whilst the placebo vaccine contained PBS in place of the antigen portion. Booster vaccinations were administered 3 weeks after the initial injection and mice were challenged with $1x10^6$ ifu, *C. psittaci*, strain S26/3, 3 weeks later.

Only low levels of chlamydial LPS were detected in either the liver or spleen of the infected control mice 6 days after challenge, at necropsy. The high variation in values for this group made statistical analysis by the Student's t-test inappropriate and therefore individual values were considered as either positive or negative and analysed by Fisher's Exact test. The negative cut-off value used was :- mean value of negative control group + 3SD. The proportion of positive mice in each group was recorded. Of the infected control mice immunised with placebo vaccine, 90% were positive for *C. psittaci* infection in the spleen. In comparison, only 30% of mice infected after vaccination with native chlamydial EBs were positive on day 6 after challenge.

In the groups vaccinated with tMOMP (either insoluble or solubilised), 50% and 67% respectively were positive in the spleen. Both vaccines therefore appeared to considerably reduce chlamydial LPS levels in the spleen. In contrast, 100% of mice vaccinated with solubilised mMOMP and 89% vaccinated with the insoluble form were positive for chlamydial LPS in the spleen. Likewise, 90% of mice vaccinated with a combination of VS1 and VS3 were positive for splenic chlamydial LPS. No

protection was therefore evident with the mMOMP construct or the VS1/VS3 combination in this model.

In a second experiment (Table 7.6b.) which used a more virulent inoculum batch, higher levels of splenic infection were observed in control mice. The range of values within this group was small and therefore analysis of data by the Student's t-test was appropriate. Using this test, mice in Group 1 (vaccinated with mMOMP19) and in Groups 2 and 3 (vaccinated with insoluble and soluble forms of tMOMP, respectively) had significantly less splenic chlamydial LPS on day 6 after infection compared with the control mice of Group 4.

**Legend for *Table 7.6. Active immunisation of mice with recombinant antigens - Model 1.***

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EB - chlamydial elementary body

VS - variable segment, produced as recombinant antigen expressed by *E. coli*

- *Groups of n mice injected subcutaneously antigen in adjuvant (Montanide ISA 50 + Alhydrogel).*
- *Mice were challenged with $1x10^6$ ifu C. psittaci, 3 weeks after second vaccination and killed on day 6 p.i.; spleens and livers assayed for chlamydial infection by capture ELISA of LPS.*
- *% +ve ; percent of mice greater than negative controls, (mean of Group 7 + 3 SD).*

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*Table 7.6. Active immunisation of mice with recombinant antigens - Model 1.*

a) Recombinant antigens mMOMP and tMOMP.

| group | vaccine | n | LPS concentration (ng/g) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | spleen | | | liver | | |
| | | | **mean** | sem | % +ve | **mean** | sem | % +ve |
| **1** | mMOMP | 9 | **70** | 16 | 89 | **28** | 6 | 89 |
| **2** | soluble mMOMP | 9 | **1383** | 1226 | 100 | **61** | 21 | 100 |
| **3** | tMOMP | 10 | **15** | 6 | 50 | **10** | 3 | 40 |
| **4** | soluble tMOMP | 9 | **44** | 17 | 67 | **28** | 12 | 67 |
| **5** | native EBs | 10 | **29** | 18 | 30 | **32** | 10 | 70 |
| **6** | placebo, infected | 20 | **275** | 239 | 90 | **12** | 2 | 70 |
| **7** | placebo, uninfected | 10 | **4** | 0 | 0 | **4** | 0 | 0 |
| **8** | VS1 + VS3 | 10 | **92** | 52 | 90 | **16** | 2 | 70 |

*Refer to previous page for legend.*

*b) Recombinant antigens mMOMP19 and tMOMP.*

| group | vaccine | n | LPS concentration (ng/g) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | spleen | | | liver | | |
| | | | **mean** | *sem* | rank | **mean** | *sem* | *rank* |
| 1 | mMOMP19 | 9 | **789** | *144* | 4 | **622** | *121* | *4* |
| 2 | tMOMP | 10 | **814** | *194* | 2 | **888** | *261* | *1* |
| 3 | soluble tMOMP | 10 | **795** | *145* | 3 | **817** | *327* | *2* |
| 4 | placebo, infected | 10 | **1245** | *155* | 1 | **770** | *99* | *3* |

- *refer to Table 7.6a. for legend.*
- *p values calculated by Student's t-test.*

**7.4.2.2. Passive Immunisation - Model 2:** Tables 7.7a and 7.7b show results of 2 experiments in which antibodies from sheep vaccinated with recombinant forms of MOMP were used to treat chlamydial inoculum prior to intraperitoneal injection into mice. Although immune serum reduced chlamydial LPS in mouse spleens significantly ($p<0.01$), compared with control mice, serum from mice vaccinated with placebo was also able to significantly reduce hepatic LPS levels ($p<0.01$). The reason for this anomaly was unknown, however results which otherwise would have been significant were considered with some caution. Nevertheless, reduced levels of LPS were found in livers from sheep vaccinated with both forms of tMOMP, native EBs and soluble mMOMP. In contrast, vaccination of sheep with the insoluble form of mMOMP resulted in similar LPS levels in mouse livers as negative control mice.

In the second experiment, serum from sheep vaccinated with placebo was not protective and LPS levels were comparable to those in negative control mice. Pre-treatment of inoculum with immune sheep serum resulted in lower levels of LPS in both the liver and spleen of mice, although the results were not significant. Likewise, sera from sheep vaccinated with either tMOMP (insoluble or soluble) or to a lesser extent mMOMP19, resulted in reduced levels of LPS.

***Table 7.7.*** *Passive immunisation of mice - Model 2.*

*a) Recombinant antigens mMOMP and tMOMP.*

| | | | chlamydial LPS (ng/g tissue) | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Group** | **n** | **vaccine/serum source** | **spleen** | | | **liver** | | |
| | | | **meann** | *sem* | n° +ve | **mean** | *sem* | n° +ve |
| 1 | 10 | mMOMP; lambs | **59** | *26* | 10 | **666** | *246* | 10 |
| 2 | 10 | soluble mMOMP; lambs | **4** | *3* | 6 | **76**** | *26* | 10 |
| 3 | 10 | tMOMP; lambs | **1**** | *1* | 2 | **22**** | *19* | 3 |
| 4 | 10 | soluble tMOMP; lambs | **0**** | *0* | 0 | **2**** | *1* | 2 |
| 5 | 10 | placebo; lambs | **3**** | *2* | 5 | **40**** | *18* | 7 |
| 6 | 10 | native EBs; lambs | **0**** | *0* | 0 | **0**** | *0* | 0 |
| 7 | 20 | immune serum; adult ewes | **0**** | *0* | 2 | **0**** | *0* | 0 |
| 8 | 10 | naive serum; adult ewes | **16** | *5* | 10 | **566** | 207 | 10 |

- *live chlamydiae ($2x10^6$ ifu/ml, titre at injection) was incubated at 37°C/45min with an equal volume of heat inactivated sheep immune or naive serum (inoculum mixture).*
- *8-14 week old mice were inoculated intraperitoneally with 1ml inoculum mixture.*
- *mice were killed on day 6 p.i.; spleens and livers assayed for chlamydial infection by capture ELISA of LPS.*

*** significantly lower by Student's t-test than naive serum (Group 8); $p<0.01$.*

***Table 7.7.*** *Passive immunisation of mice - Model 2.*

*b) Recombinant antigens mMOMP19 and tMOMP.*

| group | treatment | Chlamydial LPS (ng/g) | | | | | |
|---|---|---|---|---|---|---|---|
| | | spleen | | | liver | | |
| | | **mean** | *sem* | rank | **mean** | *sem* | rank |
| 1 | tMOMP | **420** | *115* | 2 | **196** | *44* | 4 |
| 2 | soluble tMOMP | **117** | *35* | 5 | **168** | *74* | 5 |
| 3 | mMOMP 19 | **323** | *90* | 3 | **262** | *68* | 3 |
| 4 | placebo | **1120** | *566* | 1 | **473** | *187* | 1 |
| 5 | immune serum | **79** | *14* | 6 | **108** | *31* | 6 |
| 6 | naive serum | **290** | *118* | 4 | **429** | *201* | 2 |

- refer to Table 7.7a. for legend. N.B. inoculum titre at injection was only $1x10^6$ ifu/ml.

No significant differences were apparent between the standard naive serum control (Group 6) and any of the other groups including the immune serum control ($p>0.05$).

## 7.5 Discussion

In this chapter, experiments demonstrated that sub-cutaneous immunisation of naive sheep with chlamydial antigen presented as native or as recombinant MOMP antigens, induced strong antibody responses. Western blot analysis of sera from sheep immunised with native chlamydial antigen indicated that MOMP was immunodominant compared to other chlamydial proteins. However, whereas immunisation with all three recombinant constructs (fMOMP, mMOMP and tMOMP) induced good seroconversion against MOMP, only tMOMP conferred protection against OEA in pregnant sheep. Clearly, detection of seroconversion post-vaccination was unable to distinguish protective from non-protective constructs. Only some epitopes of the whole MOMP molecule therefore appear to be involved in protection. Similarly, *C. trachomatis* infections in humans can induce seroconversion without conferring protection. In fact, some of these antibody responses are detrimental to the host, in particular those occurring against the heat shock proteins, hsp 60 and hsp 70 (Watkins *et al.* , 1986; Taylor *et al.*, 1990; Brunham *et al.*, 1992).

Considering each arm of the immune system separately, T-cell involvement in protection was assessed using plasma from immunised animals. When PBLs were stimulated *in vitro* with native chlamydial EBs, those which had originated from convalescent ewes, or ewes immunised with either purified or MOMP-enriched vaccines were able to produce IFN-γ. This strongly indicated priming of T-cell epitopes *in vivo* by various forms of MOMP which could subsequently be recognised *in vitro* by MOMP in its native configuration. Stimulation of PBLs also occurred, although to a lesser extent, when the immunising antigen comprised fMOMP or mMOMP (soluble), again indicating recognition of T-cell epitopes common to both these recombinant constructs and chlamydial EBs. The IFN-γ response was more vigorous after substituting the native antigen (whole EBs) for a solubilised, purified form of MOMP. This preparation had been shown to protect sheep against *C. psittaci* infection (Tan *et al.,* 1990) and was therefore considered to be an

appropriate form against which to compare recombinant MOMP constructs. The greater production of IFN-γ in response to purified MOMP as opposed to whole EBs may simply reflect a higher MOMP concentration in the purified preparation. IFN-γ production by PBLs from sheep vaccinated with recombinant MOMPs implies that these antigens share common T-cell epitopes with purified, native MOMP. However, IFN-γ was released by ovine PBLs *in vitro* irrespective of whether the immunising antigen protected sheep against OEA. Therefore, it was necessary to use a more discerning assay to select protective recombinant antigens than *in vitro* stimulation of T-cells.

Some correlation between protection against *C. psittaci* infection *in vivo* and *in vitro* was evident using the SNA. The results were viewed with caution however, and the unreliable nature of the *in vitro* assay taken into account. Chlamydial infection of McCoy cells appeared to be reduced by over 80% in the SNA using sera from sheep vaccinated with tMOMP. A similar degree of neutralisation occurred using sera from convalescent, post-abortion sheep. In contrast, although some neutralisation was evident with sera from fMOMP-vaccinated sheep, the effect was much less. A vaccine preparation of fMOMP failed to protect sheep against experimental OEA. Taken together, these results suggest that although overall seroconversion did not represent vaccine efficacy, the SNA may be capable of differentiating between sera which contain neutralising antibodies and sera which do not.

Due to the difficulties encountered with the SNA throughout vaccine efficacy trials, it became apparent that mouse models would be the major tool by which to screen recombinant MOMP constructs. Earlier work had demonstrated that CBA/ADRA mice were receptive to vaccination with chlamydial EBs and strong MOMP-specific antibody responses had been detected (G. E. Jones, personal communication). In contrast, the current study found only limited antibody response after vaccination of mice with recombinant proteins. An attempt during the current study to produce hyperimmune serum against tMOMP (personal observation) required 3 vaccinations to elicit a detectable antibody response in ADRA/CBA mice. Thus, there appeared to

be a major difference between sheep and CBA/ADRA mice in ability to respond immunologically to epitopes on the recombinant proteins. Interestingly, this did not appear to be a total inability of the mice to produce MOMP-specific antibodies, since native MOMP was stimulatory, as was recombinant VS1 combined with VS3 in a vaccine formulation. The mice were assumed therefore, not to be deficient in MOMP-specific B-cells. In addition, the antigen dose used in mice was one tenth that used to immunise sheep and should have been sufficient to induce a good antibody response. Since recombinants of VS1 and VS3 stimulated antibody production, it appeared likely that T-helper epitopes may be located within one or both of these regions. The apparent difference in induction of a humoral immune response by peptides of MOMP in comparison to recombinant MOMP protein, may be explained by elucidation of the processing and MHC class-II presentation of chlamydial antigens. Indeed, presentation of vaccine as peptide rather than protein may represent an important pre-processing of the recombinant construct. Leclerc *et al.* (1989) reported a considerable increase in antibody response to a recombinant peptide, after pre-processing of the bacterial preparation compared to immunisation of mice with the live bacteria. This supports the presentation of a chlamydial vaccine as an amalgamation of peptides which have been found to have both T-cell and B-cell activity. With respect to the likely involvement of MHC class-II, T-helper cell activity has also been reported for *C. trachomatis* (Su *et al.,* 1990; Allen, Locksley and Stephens, 1991; Stagg *et al.,* 1993). Evidence suggests that T-helper cell epitopes may be located in the VS3 region of MOMP, inducing B-cell clones to produce neutralising antibodies specific to VS1 and VS4. An alternative explanation for the apparent difference in induction of seroconversion between recombinant MOMP proteins and peptides in mice can not be ignored. This may be manifest as a loss of speculative T-cell suppressor epitopes in peptide preparations.

In general, some protection against chlamydial colonisation of mouse tissues was evident after immunisation with either recombinant tMOMP or with mMOMP19. Protection was apparent in both active immunisation of mice (Model 1) and passive transfer of serum from vaccinated sheep to naive mice (Model 2). A major problem

with the mouse models however, remained the variable infection levels of organs (liver and spleen) obtained after intraperitoneal inoculation with live chlamydiae. In some experiments low rates of infection made determination of protection difficult, whilst in others, large variations in infection levels of mice resulted in statistically invalid results. In other studies, although organ infections varied to some extent between mice, the effect was insufficient to affect statistical analysis of the data (Buzoni-Gatel and Rodolakis, 1983; Rodolakis *et al.,* 1989; Buzoni-Gatel *et al.,* 1990). The reason for the discrepancy between the results presented here and those of other workers is unknown, although in the case of the latter citation, Swiss White out-bred mice were used rather than CBA. Accordingly, the extent of effect of mouse strain on the outcome of vaccine efficacy trials may be significant. There is evidence that both susceptibility and antibody response to chlamydial infection is MHC-linked (Fuentes and Orfila, 1989; Tuffrey *et al.,*1992; Zhong and Brunham, 1992; Buzoni-Gatel *et al.,* 1994; Qu *et al.,* 1994). H-$2^k$ haplotypes such as the CBA strain have been categorised as low-MOMP responders, although alternative pilot studies indicated that CBA/ADRA in fact gave a higher response (G.E. Jones, personal communication). In view of this, two factors, namely epitope recognition and the resulting production of protective antibodies in mice are by-passed in the passive immunisation model (Model 2). Furthermore, the mechanisms by which chlamydiae are neutralised in the SNA and by Model 2 (passive immunisation of mice) may be found to be similar, however the exact processes remain unclear. Evidence in this chapter suggests that protective recombinant antigen preparations can be selected on the basis of their performances in the mouse models developed here, with tentative confirmation arising from the application of the SNA. Presentation of the selected antigens as a vaccine preparation however, requires further investigation in order that the most effective immune response be mounted in sheep.

# CHAPTER 8

## IDENTIFICATION OF A PROTECTIVE, LINEAR, B-CELL EPITOPE ON VARIABLE SEGMENT 2 OF MOMP

## 8.1. Introduction

It has been shown that, in general, there is no relationship between antibody titres and immunity to chlamydial diseases. In particular, individuals exposed to the 57-60kDa heat-shock protein of *C. trachomatis* (Morrison *et al.,* 1989; Yuan *et al.,* 1992) show signs of a hypersensitivity reaction which causes chronic long-term damage to the host (Grayston *et al.,* 1971; Taylor-Robinson and Ward, 1989). A similar occurrence has not, as yet, been demonstrated with *C. psittaci* infection, except for guinea pig inclusion conjunctivitis (GPIC). This strain of *C. psittaci* is often used as an experimental model for *trachoma* given the similarities in associated immune responses between GPIC and biovars A, B and C of *C. trachomatis*. Not only is it essential therefore, to differentiate protective epitopes from harmful ones in order to reduce the occurrence of adverse reactions to chlamydial vaccines, but elucidation of such epitopes will lead to a greater understanding of the pathogenic mechanisms underlying chlamydial infections.

This chapter describes ovine antibody profiles with respect to MOMP and variable segments 1 and 2 (VS1 and VS2). In addition, pools of polyclonal antibodies against recombinant VS1 and VS2 were prepared from post-abortion sheep serum by affinity column purification and were subsequently investigated for their neutralising and immunopathological properties using the passive immunisation mouse model described in Chapter 4. Corroborative evidence for the observed results was obtained using a VS2-specific monoclonal antibody.

## 8.2. Antibody profiles in sheep vaccinated with recombinant antigens or experimentally infected with *C. psittaci.*

### 8.2.1. Materials and Methods.

#### 8.2.1.1. Serum sources

Serum samples were obtained from 4-6 year old, female, Blackface/Swaledale sheep, from a flock certified free of abortifacient *C. psittaci* under the Premium Sheep Health Scheme (Scotland). Groups of sheep were vaccinated with either:-

a) insoluble tMOMP (batch 1),
b) insoluble tMOMP (batch 2), or
c) mMOMP-19.

Six blood samples were collected from each animal over 12 months, during which time the sheep had been successfully mated and subsequently challenged with live, abortifacient *C. psittaci.* The diagram in Fig. 8.1. illustrates the serum sample collection times in relation to other events during the experimental period.

#### 8.2.1.2. ELISA antigens

Each serum sample was tested by ELISA for immunoglobulin isotype using anti-ovine IgG(1+2)-specific and IgG2 specific polyclonal antibodies (Serotec).

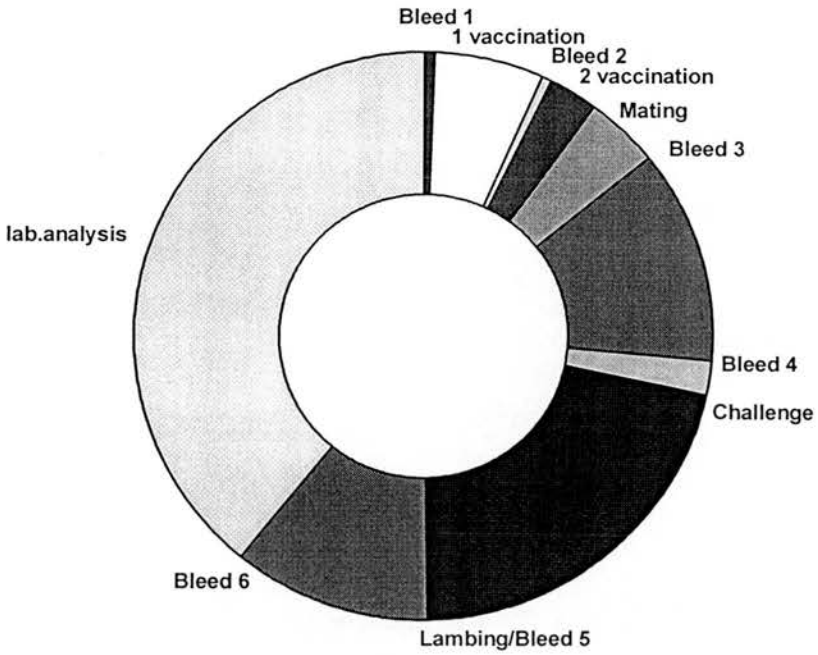



*Fig. 8.1. Calendrical schedule of events in a typical sheep vaccination/lambing experiment. (scale; 360° = 12 months)*

In addition, tMOMP sera were assayed against 3 different antigens. These were:-

i) semi-purified chlamydial elementary bodies,

ii) 16 amino acid recombinant peptide (GST constuct) representing variable segment one (VS1) of MOMP, and

iii) 16 amino acid recombinant peptide (GST construct) representing variable segment two (VS 2) of MOMP.

Preparation methodologies for the ELISA antigens can be found in section 2.5.1.

### 8.2.2. Results.

#### 8.2.2.1. Determination of serum antibody isotypes and correlation with subsequent protection against abortion.

Both IgG1 and IgG2 contents were analysed by an indirect ELISA procedure. The use of (rabbit)anti-sheep, IgG2-specific reagent (Serotec) allowed the determination of IgG2 antibody levels in serum samples. IgG1 antibody levels were calculated by subtraction of IgG2 titres from total detected IgG. Although control sera from uninfected ewes contained moderate levels of circulating IgG2 antibodies, levels of chlamydia-specific IgG2 antibodies in the test sera or chlamydia-positive control sera were below the level of detection in this particular assay. High titres of IgG1 were however detected in the majority of post-vaccination samples, in which levels varied depending on the vaccine antigen used to immunise sheep. Sheep were categorised as having aborted due to OEA based on several criteria:- a) lambs born either dead or dying within 48h of birth with no other obvious cause, b) gross placental lesions typical of OEA apparent c) shorter gestation period than uninfected control animals and d) demonstration of chlamydiae at abortion in placental/vaginal samples.

**i) Vaccination with tMOMP, batch 1.**

Table 8.1. shows antibody titres post-vaccination with batch 1 tMOMP. Grouping sheep into those which aborted and those which lambed, indicated that antibody titres were lower post-vaccination (Days 21, 42 and 70) in animals which aborted, compared to those which lambed. Statistical analysis revealed no significance using the Student's t-test, although the trend appeared to be relatively strong.

**ii) Vaccination with mMOMP-19.**

mMOMP-19 which provided no significant protection against abortion, induced varying antibody titres to MOMP (Table 8.2.). The predominant isotype was again

IgG1. Again, antibody titres pre-challenge were lower in ewes which subsequently aborted, compared to those which lambed, although the trend appeared to be much weaker than with tMOMP. Antibody titres at parturition however, did not appear to follow any trend either in ewes which aborted, or those which lambed. No statistical significance could be attributed to this data.

**iii) Lambing/abortion antibody titres.**

As with the above data, only chlamydia-specific IgG1 antibodies could be detected in sera taken from ewes at the time of lambing or abortion (bleed 5). Furthermore, unvaccinated, positive control animals responded to abortion by producing solely IgG1 antibodies in detectable quantities. IgG1 antibodies to *C. psittaci* persisted up to 5 weeks post-abortion (bleed 6).

Immunoblots of sera from ewes at lambing/aborting (Fig. 8.2) illustrate the difference in response to vaccination between recombinant tMOMP and mMOMP19. When compared to Fig. 8.2, the top panel represents responses to tMOMP (regularly immunogenic), while the lower panel represents mMOMP19 (irregularly immunogenic). Both immunoblots used urografin purified chlamydial antigen (S26/3) as the coating antigen.

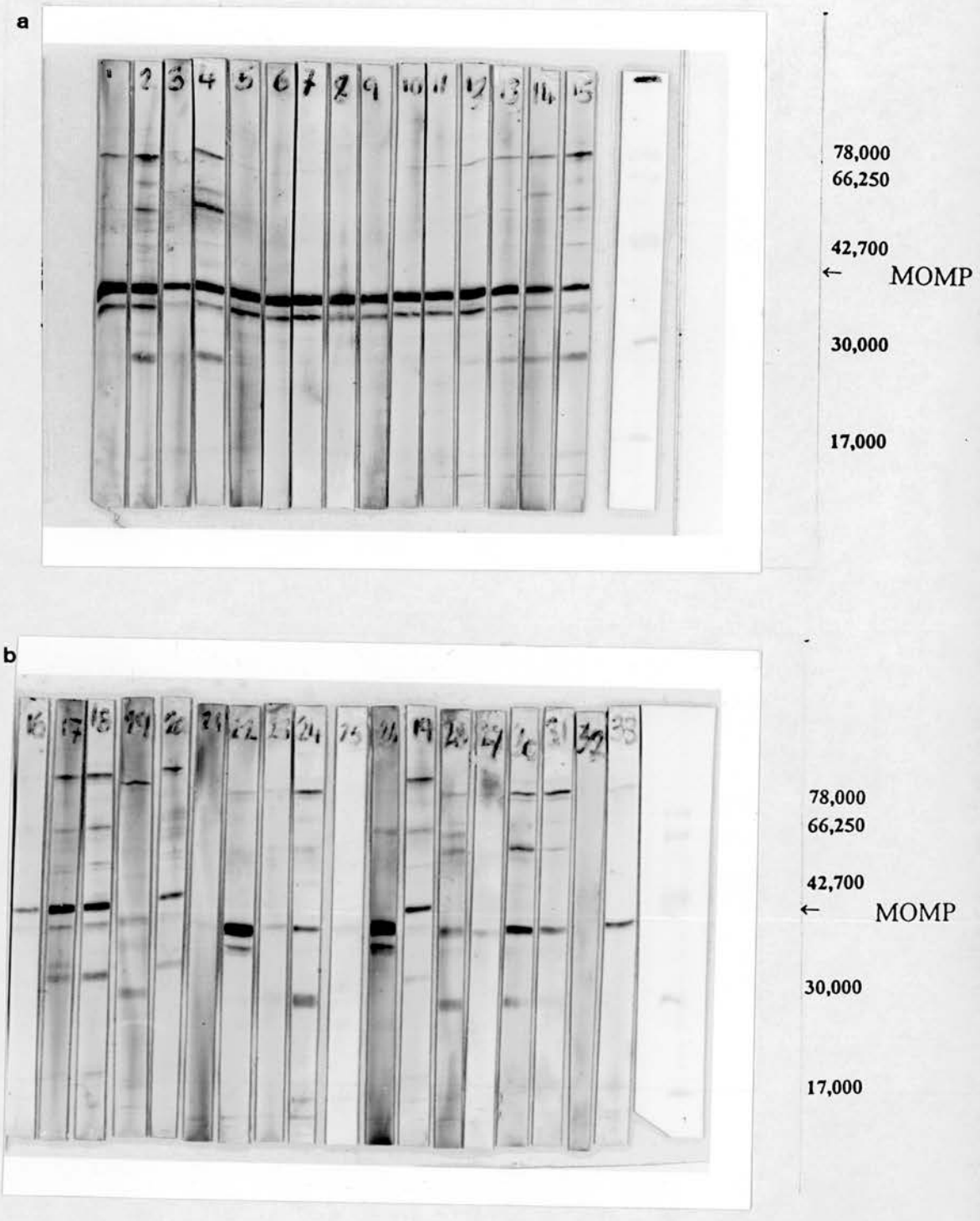



Fig 8.2. Immunoblots of sera from ewes at lambing/aborting, blotted against urografin purified chlamydial antigen (S26/3)

a) tMOMP vaccination, b) mMOMP19 vaccination

### 8.2.2.2. Specificity of antibodies to VS1 and VS2 peptides of MOMP.

#### i) Vaccination with tMOMP, batch 1.

Antibodies specific for VS1 (Table 8.3.) and VS2 (Table 8.4.) were measured using an ELISA specifically developed for this purpose (Materials and Methods, Section 2.6.1.). Comparing the specificities of antibodies produced, it was apparent that VS1-specific antibodies were produced in higher concentrations than VS2-specific antibodies (means of Tables 8.3. and 8.4.).

All animals seroconverted well to VS1 (Table 8.3). However, antibody titres pre-challenge were lower in ewes which subsequently aborted, compared to those which lambed. In contrast, antibody titres at parturition were not greatly different in ewes which aborted, compared to those which lambed, all sheep seroconverting well to VS1 after challenge (100 dpv),

Titres of VS2-specific antibodies pre-challenge (Table 8.4), were also considerably lower in ewes which subsequently aborted, compared to those which lambed . In this case, statistical significance was evident ($p< 0.01$). Conversely, seroconversion to VS2 was undetectable at 100 dpv (post-challenge) and antibody titres at parturition again appeared to be similar in ewes which aborted and those which lambed.

***Table 8.1.*** *Levels of serum IgG1 produced in response to vaccination of sheep with 100μg/ml tMOMP, batch 1*[1].

| Sheep | Antibody Titre ($OD_{492}$) | | | | | Clinical History |
|---|---|---|---|---|---|---|
| | 21 dpv | 42 dpv | 70 dpv | bleed 5[2] | bleed 6[3] | |
| W2710 | 0.014 | 0.034 | 0.077 | 1.647 | 1.992 | A |
| W2722 | 0.409 | 1.453 | 1.541 | 0.189 | 0.466 | L |
| X2754 | 0.219 | 0.277 | 0.335 | 1.146 | 1.093 | A |
| X2756 | 0.064 | 1.372 | 0.494 | 0.848 | 0.686 | L |
| X2757 | 0.912 | 0.432 | 0.826 | 0.199 | 0.668 | L |
| X2782 | 0.334 | 1.142 | 1.018 | 0.264 | 0.410 | L |
| X2784 | 0.007 | 0.384 | 0.256 | 0.065 | 0.331 | L |
| X2786 | 0.003 | 0.295 | 0.680 | 0.175 | 0.280 | L |
| X2789 | 1.675 | 0.620 | 1.697 | 0.544 | 0.063 | L |
| X2798 | 0.153 | 1.274 | 1.642 | 0.547 | 0.918 | L |
| X2799 | 1.867 | 0.805 | 0.925 | 0.381 | 0.503 | L |
| X2876 | 0.023 | 0.994 | 0.415 | 0.316 | 1.022 | L |

**A, aborted ; L, lambed**

[1] *sheep vaccinated with 2x1ml doses; 1° vaccination at 14 weeks pre-challenge, 2° vaccination at 11 weeks pre-challenge, challenged at 10 weeks (70days) gestation.*

[2] *sheep bled at time of lambing/aborting*

[3] *sheep bled 5 weeks (approximately) after lambing/aborting*

***Student's t-test:*** *Antibody titres pre-challenge were not significantly lower in ewes which subsequently aborted, compared to those which lambed ($p>0.05$).*
*Antibody titres at parturition were not significantly higher in ewes which aborted, compared to those which lambed ($p>0.05$).*

***Table 8.2.*** *Levels of serum IgG1 produced in response to vaccination of sheep with 100μg/ml mMOMP-19*[1].

| Sheep | Antibody Titre ($OD_{492}$) | | | | | Clinical History |
|---|---|---|---|---|---|---|
| | 21 dpv | 42 dpv | 70 dpv | bleed 5 | bleed 6 | |
| W3057 | 0.010 | 0.673 | 1.059 | 0.729 | 0.646 | A |
| X2768 | 0.720 | 0.842 | 0.550 | 0.532 | 0.508 | L |
| X2772 | 1.127 | 1.393 | 1.544 | 1.035 | 1.116 | L |
| X2774 | 0.084 | 0.340 | 0.668 | 0.704 | 0.626 | A |
| X2775 | 0.156 | 0.596 | 0.416 | 0.273 | 0.696 | A |
| X2781 | 0.692 | 1.042 | 1.054 | 0.644 | 0.000 | A |
| X2787 | 0.040 | 0.638 | 0.978 | 0.081 | 0.215 | L |
| X2788 | 0.400 | 0.669 | 0.799 | 0.398 | 0.000 | L |
| X2797 | 0.256 | 0.676 | 0.568 | 0.238 | 0.298 | L |
| X2837 | 0.008 | 0.118 | 0.685 | 0.899 | 0.740 | A |
| X2852 | 0.356 | 1.082 | 0.621 | 0.292 | 0.232 | L |
| X2854 | 0.217 | 0.613 | 0.346 | 0.554 | 1.017 | L |
| X2873 | 0.106 | 0.482 | 0.140 | 0.562 | 0.740 | A |
| X2885 | 0.702 | 1.240 | 0.876 | 0.590 | 0.490 | L |
| X2923 | 0.062 | 0.090 | 0.108 | - | - | A |

*refer to Table 8.1 for legend*

***Student's t-test:*** *Antibody titres pre-challenge were not significantly lower in ewes which subsequently aborted, compared to those which lambed ($p>0.05$).*
*Antibody titres at parturition were not significantly different in ewes which aborted, compared to those which lambed ($p>0.05$).*

***Table 8.3.*** *Levels of VS1-specific antibodies in sera from sheep vaccinated with 100μg/ml tMOMP, batch 1.*

| Sheep | Antibody Concentration ($OD_{492}$) | | | | | Clinical history |
|---|---|---|---|---|---|---|
| | 21 dpv | 42 dpv | 70 dpv | bleed 5 | bleed 6 | |
| **W2710** | 0.014 | 0.303 | 0.090 | 1.350 | 1.427 | A |
| **W2722** | 1.408 | 1.569 | 1.641 | 0.681 | 0.571 | L |
| **X2754** | 0.327 | 0.725 | 0.704 | 1.694 | 1.653 | A |
| **X2756** | 1.232 | 1.337 | 1.626 | 1.552 | 1.480 | L |
| **X2757** | 1.465 | 1.514 | 1.590 | 1.315 | 1.383 | L |
| **X2782** | 1.339 | 1.418 | 1.407 | 1.348 | 1.160 | L |
| **X2784** | 0.746 | 1.413 | 0.984 | 0.908 | 1.085 | L |
| **X2786** | 0.034 | 0.245 | 1.350 | 0.964 | 0.901 | L |
| **X2789** | 1.668 | 1.624 | 1.681 | 1.326 | 1.053 | L |
| **X2798** | 1.313 | 1.523 | 1.585 | 1.336 | 1.425 | L |
| **X2799** | 1.774 | 1.573 | 1.675 | 1.327 | 1.207 | L |
| **X2876** | 1.458 | 1.498 | 1.284 | 1.389 | 1.423 | L |

[1-4] *see Table 8.1 for legend.*

[6] *sheep bled approximately 6 weeks after bleed 5.*

***Student's t-test:*** *Antibody titres pre-challenge were not significantly lower in ewes which subsequently aborted, compared to those which lambed ($p>0.05$).*
*Antibody titres at parturition were not significantly different in ewes which aborted, compared to those which lambed ($p>0.05$).*

***Table 8.4.*** *Levels of VS2-specific antibodies in sera from sheep vaccinated with 100μg/ml tMOMP, batch 1.*

| | Antibody Concentration ($OD_{492}$) | | | | | Clinical |
|---|---|---|---|---|---|---|
| **Sheep** | **21 dpv** | **42 dpv** | **70 dpv** | **[5]bleed 5** | **[6]bleed 6** | **History** |
| **W2710** | 0.012 | 0.049 | 0.019 | -0.091 | 1.031 | A |
| **W2722** | 1.377 | 1.414 | 1.455 | 0.124 | 0.178 | L |
| **X2754** | 0.024 | 0.106 | 0.005 | 0.001 | -0.197 | A |
| **X2756** | 0.497 | 1.008 | 0.121 | -0.010 | -0.008 | L |
| **X2757** | 0.120 | 1.083 | 0.628 | 0.036 | 0.058 | L |
| **X2782** | 0.972 | 1.511 | 1.368 | 0.621 | 0.329 | L |
| **X2784** | 0.035 | 0.567 | 0.132 | 0.014 | 0.019 | L |
| **X2786** | 0.022 | 0.020 | 0.072 | 0.014 | 0.112 | L |
| **X2789** | 1.326 | 0.641 | 1.218 | -0.009 | 0.002 | L |
| **X2798** | 1.556 | 1.583 | 1.600 | 0.847 | 0.046 | L |
| **X2799** | 1.012 | 0.953 | 0.470 | 0.029 | 0.016 | L |
| **X2876** | 0.426 | 0.727 | 0.157 | 0.004 | 0.127 | L |

[1-6] *see table 8.3. for legend.*

***Student's t-test:*** *Antibody titres pre-challenge were not significantly lower in ewes which subsequently aborted, compared to those which lambed ($p<0.001$).*
*Antibody titres at parturition were not significantly different in ewes which aborted, compared to those which lambed ($p>0.05$).*

## 8.3. Neutralising abilities of VS1- and VS2-specific polyclonal antibodies *in vivo.*

### 8.3.1. Materials and Methods.

#### 8.3.1.1. Preparation of affinity purified polyclonal antibodies.

Affinity purified antibodies were prepared from a pool of post-abortion sheep serum. Briefly, each variable segment was prepared as a recombinant peptide (Section 2.7.2.) and individually coupled to cyanogen bromide-activated sepharose beads at pH 7.0. Immune serum was passed slowly over each column (1ml/min) and adsorbed antibodies removed by reducing the pH to between pH2-

5. A second antibody fraction was eluted from each column using elution buffer + 6M urea. The pH of each fraction was neutralised using 1M Tris. The fractions eluted under mild conditions were termed "low affinity" antibodies, whilst those eluted with 6M urea in the elution buffer, "high affinity antibodies". All fractions were analysed for peptide specificity by immunoblotting and quantified by indirect ELISA.

#### 8.3.1.2. *In vivo* neutralisation.

Four pools of affinity purified antibodies were formed as follows:-

Pool 1 - VS1-specific "low affinity" antibodies
Pool 2 - VS1-specific "low affinity" + "high affinity" antibodies
Pool 3 - VS2-specific "low affinity" antibodies
Pool 4 - VS2-specific "low affinity" + "high affinity" antibodies

Concentrations of specific antibodies were adjusted to equivalent levels to those in the original whole serum i.e. 0.67mg/ml VS1, 0.37mg/ml VS2. However, because the ratio of "high" to "low" affinity antibodies in whole serum was unknown, each fraction was adjusted to the total concentration determined for each peptide in whole immune serum. This should have allowed for excess amounts of antibody except in pool 2, where a low recovery rate of "high affinity" antibodies against VS1 meant that this criterion could not be met in full.

Two experiments were performed using the passive immunisation mouse model (Chapter 4). In the first experiment, Pools 1 and 3 were examined for their abilities to reduce liver and spleen colonisation with live *Chlamydia*. Pools 2 and 4 were tested in the second experiment.

### 8.3.1.2.1. Designs of experiments.

#### a) Experiment 1.

*Table 8.5. Design of experiment 1.*

| Group | n | Inoculation *(C. psittaci)* | Antibody Source |
|---|---|---|---|
| 1 | 5 | + | "low affinity" VS1-specific antibodies |
| 2 | 5 | + | "low affinity" VS2-specific antibodies |
| 3 | 11 | + | immune sheep serum |
| 4 | 12 | + | naive sheep serum |
| 5 | 6 | - | - |

Inoculation mixtures contained 1 x$10^6$ ifu/ml *C. psittaci.*.

#### b) Experiment 2.

*Table 8.6. Design of experiment 2.*

| group | n | *Inoculation (C. psittaci)* | Antibody Source |
|---|---|---|---|
| 1 | 9 | + | "high"+ "low" affinity VS1-specific antibodies |
| 2 | 9 | + | "high"+ "low" affinity VS2-specific antibodies |
| 3 | 10 | + | immune sheep serum |
| 4 | 10 | + | naive sheep serum |
| 5 | 4 | - | - |

### 8.3.2. Results.

#### a) Experiment 1.

The inoculum titre used was half the optimal, due to diminishing stocks of inoculum. The effect of using a sub-optimal titre is shown by the lack of infection in Group 4 (*C. psittaci* + naive serum) (Table 8.8.). Nevertheless, a statistically significant difference was apparent in liver infection levels control Groups 3 and 4 ($p<0.01$); there was no difference between splenic infection levels ($p>0.05$). Both groups treated with "low affinity" antibodies against VS1 or VS2 (Groups 1 and 2, respectively), yielded enhanced infection levels in both liver and spleen ($p<0.01$).

*Table 8.7. Experiment 1. Protection afforded by low affinity antibodies against VS1 and VS2.*

| | | | Chlamydial LPS (ng/g) | | | |
|---|---|---|---|---|---|---|
| **Group** | **n** | **Antibody Source** | **liver** | | **spleen** | |
| | | | **mean** | ***sem*** | **mean** | ***sem*** |
| 1 | 5 | "low affinity" VS1-specific | **293** | *76* | **1872** | *687* |
| 2 | 5 | "low affinity" VS2-specific | **288** | *58* | **781** | *80* |
| 3 | 11 | immune sheep serum | **25** | *4* | **70** | *8* |
| 4 | 12 | naive sheep serum | **102** | *12* | **83** | *6* |
| 5 | 6 | non-infected control | **0** | *0* | **0** | *0* |

**Statistical analysis (Student's t-test):-**

- Group 3 significantly protected ($p<0.01$) in liver, but not spleen, compared with Group 4.
- Groups 1 and 2 showed significantly higher infection in both liver and spleen compared to Group 4 ($p<0.01$).

**b) Experiment 2.**

Infection levels in Group 4 (naive serum) were significantly higher than Group 3 (immune serum) in both liver and spleen ($p<0.01$) (Table 8.9.). Unlike previous experiments with this inoculum at the same titre ($2x10^6$ ifu/ml), splenic infection levels were considerably higher than hepatic ($p<0.01$). The reason for this anomaly was unclear. The liver results indicated that although there was a trend towards lower infection in Group 1 and 2, ("high and low affinity" antibodies against VS1 and VS2, respectively), the data were not statistically significant. In contrast, infection levels in the spleen were significantly reduced in both Groups 1 (pooled VS1 antibodies) and 2 (pooled VS2 antibodies), ($p<0.05$ and $p<0.001$, respectively).

## 8.4. *In vivo* protection by chlamydia-specific monoclonal antibodies.

A panel of murine monoclonal antibodies against *C. psittaci* was tested in a passive immunisation protocol. Passive transfer of inoculation mixtures containing *C. psittaci* pre-treated with mouse monoclonal antibodies (Mabs) was carried out in a standard passive immunisation experiment (See Chapter 4). Several modifications were necessary due to the use of ascitic fluid rather than sheep serum; these are described at the appropriate places.

### 8.4.1. Sources of monoclonal antibodies.

*C. psittaci* specific MAbs (Table 8.10) were used in the form of filtered, unpurified, ascitic fluid collected from mice.

***Table 8.8.*** *Experiment 2. Protection afforded by pooled ("high and low affinity") antibodies against VS1 and VS2.*

| | | | Chlamydial LPS (ng/g) | | | |
|---|---|---|---|---|---|---|
| **Group** | **n** | **antibody source** | **liver** | | **spleen** | |
| | | | **mean** | ***sem*** | **mean** | ***sem*** |
| 1 | 9 | pooled VS1-specific antibodies ("high" + "low" affinity) | **281** | *31* | **757** | *66* |
| 2 | 9 | pooled VS2-specific antibodies ("high" + "low" affinity) | **253** | *43* | **509** | *82* |
| 3 | 10 | immune sheep serum | **0** | *0* | **13** | *4* |
| 4 | 10 | naive sheep serum | **347** | *44* | **950** | *52* |
| 5 | 4 | non-infected control | **0** | *0* | **0** | *0* |

**Statistical analysis (Student's t-test):-**

- Groups 1 and 2 showed significantly lower levels of splenic infection compared to Group 4 ($p<0.05$; $p<0.001$, respectively).
- Group 2 significantly lower than Group 1 ($p<0.01$) for spleen infection levels.
- Control groups (3 and 4) significantly different from each other ($p<0.01$) in both spleen and liver infection levels.
- No significant reduction in hepatic infection in Groups 1 and 2.

***Table 8.9.*** Properties of mouse monoclonal antibodies against abortifacient *C. psittaci* (strain S26/3).

| MAb | Isotype | Specificity | Conc$^{n.}$ (mg/L) |
|---|---|---|---|
| 13/5 | IgG3 | LPS | 493 |
| 4/11 | IgG2b | VS2 | 33 |
| 4/17 | IgG3 | VS2 | >1000 |
| 4/35 | ? | MOMP (not VS2) | 428 |
| 4/40 | IgG2a | MOMP (not VS2) | >1000 |

### 8.4.2. Inoculation mixtures.

Heat inactivated ascitic fluids contributed 25% of the final inoculation mixture volume. Guinea pig serum was included at 10% final volume and chlamydia inoculum titres were $2x10^6$ ifu/ml. As before, each mouse received 1ml inoculation mixture i.p. and was killed on day 6 p.i..

### 8.4.3. Results.

Only one MAb (4/11) significantly ($p<0.01$) reduced infection levels in liver and spleen tissue (Fig. 8.3.). This MAb was VS2 specific and of IgG2b isotype. A second anti-VS2 MAb (4/17), did not show any protective capacity. The isotype of this MAb was IgG3. No other MAbs reduced chlamydial infection to a significant degree. A repeat experiment restricted to MAb 4/11 confirmed the protective capacity of this VS2-specific monoclonal antibody.

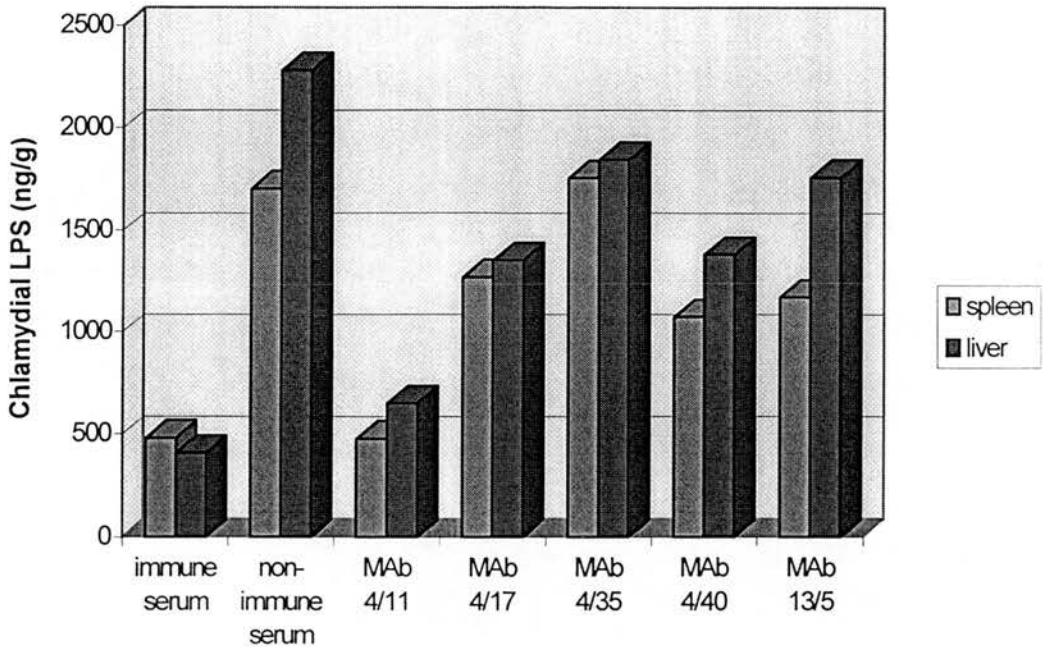



***Fig. 8.3.*** *Infection levels in the spleen and liver of mice passively immunised with monoclonal antibodies against* C. psittaci.

*Immune sera and Mab 4/11 - infection in liver and spleen significantly lower than control (non-immune sera); $p<0.01$ (Student's t-test).*

## 8.5. *In vitro* competition between MAb 4/11 and affinity purified antibodies against VS1 and VS2.

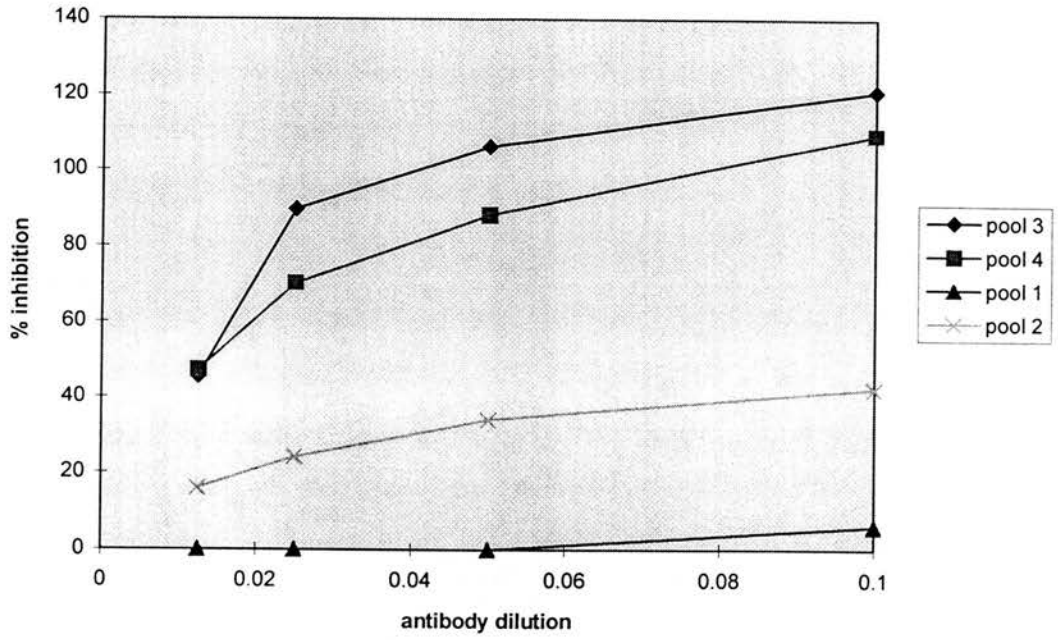
### 8.5.1. Materials and Methods.

The previously described pools of affinity purified polyclonal antibodies extracted from sheep post-abortion serum, formed the test samples in this assay. An indirect ELISA procedure (Anderson, unpublished) was used to assess the specificity of these antibodies against VS2 and VS1 using the anti-VS2 MAb, 4/11. Briefly, sarkosyl-treated chlamydial elementary bodies were used to detect chlamydia-specific antibodies in the test sample. After incubation, horseradish peroxidase-conjugated MAb 4/11 was added. Inhibition of binding of the conjugated MAb was indicated as a reduction in optical density after addition of substrate. Pools were as follows:-

Pool 1 - VS1-specific "low affinity" antibodies
Pool 2 - VS1-specific "low affinity + high affinity" antibodies
Pool 3 - VS2-specific "low affinity" antibodies
Pool 4 - VS2-specific "low affinity + high affinity" antibodies

### 8.5.2. Results.

At the lowest antibody dilution tested (1/10), both pools 3 and 4 inhibited binding of MAb 4/11 by 100% (Fig. 8.3) and significant inhibition ($p<0.01$) occurred even at a dilution of 1/80. The difference between these groups at 1/80 antibody dilution was not significant ($p>0.05$). Pool 2 ("high and low affinity" VS1-specific antibodies) caused a small, but significant ($p<0.01$) degree of inhibition. The inhibition induced was one third of that caused by pools 3 and 4 at a 1 in 80 dilution.




pool 1 - "low affinity" antibodies specific to VS1.

pool 2 - "low and high affinity" antibodies specific to VS1.

pool 3 - "low affinity" antibodies specific to VS2.

pool 4 - "low and high affinity" antibodies specific to VS2.

***Fig. 8.4.*** *Inhibition of binding of MAb 4/11 to native chlamydial antigen by polyclonal sera specific for either VS1 or VS2.*

### 8.6. Summary of results.

Comparatively moderate levels of both IgG1 and IgG2 antibodies were detected in normal sheep serum. In contrast, the predominant anti-chlamydia antibody isotype detected in sheep sera after clinical OEA was IgG1. Low levels of IgG2 antibodies may have been present in the sera, however these were not detected by the assay.

Vaccination of sheep with a protective recombinant form of MOMP (tMOMP) resulted in high levels of chlamydia-specific IgG1 in at least one of the three pre-challenge bleeds in each animal which lambed (n=10). In contrast, neither of the two ewes which aborted showed comparable levels of seroconversion in pre-challenge serum samples. This was also evident with the mMOMP19 vaccine, however seroconversion post-challenge appeared to differ between the two recombinant vaccines. Sheep that aborted and those that lambed did not produce vastly different antibody responses post-challenge when vaccinated with mMOMP19. However, they were considerably different when tMOMP was the immunising antigen. Unlike tMOMP, seroconversion to mMOMP19 appeared to be less frequent and although there was some correlation between seroconversion and lambing, both protective and non-protective epitopes appeared to have been recognised in the recombinant construct, as indicated by the random production of antibodies post-challenge. In comparison, vaccination with tMOMP appeared to prime for antibodies which would recognise native MOMP and which were mainly protective.

Vaccination of sheep with recombinant antigens of MOMP, also resulted in high levels of seroconversion to VS1 and VS2. In a group of twelve ewes, two which showed lower seroconversion in the three pre-challenge samples both aborted. Lambing was accompanied by at least one high titred serum sample to both peptides, pre-challenge (9 out of 10 cases), with one exception in which lambing occurred despite only low levels of seroconversion to VS2. Notably, neither of the sheep which aborted responded to the VS2 of tMOMP at time of abortion. In contrast, one of these animals produced VS2-specific antibodies by 6 weeks post-abortion,

indicating recognition of native VS2 following experimental infection with *C. psittaci*. The second animal remained negative for VS2-specific antibodies in the convalescent phase. Both animals seroconverted to VS1 at time of aborting, with increased titres apparent in the convalescent phase. It was notable that no seroconversion to recombinant VS2 occurred at lambing in sheep vaccinated with tMOMP, indicating that native and recombinant MOMP may differ at the VS2 site. Furthermore, while sheep W2710 appeared able to recognise native, but not recombinant VS2, sheep X2754 failed to respond to either form of VS2.

Haplotypes (MHC classes), have been shown to be important in inbred mouse strains with respect to immune responses to MOMP. It may also be that the response of sheep to infection with *C. psittaci* after vaccination with various forms of MOMP is similarly controlled by haplotype. The responsiveness to MOMP peptides, in particular VS2, would be of major interest following the initial results reported here.

A pattern of seroconversion following infection was identified in which sheep responded by producing chlamydia-specific IgG1 antibodies. The population of antibodies generated, comprised high titres of antibodies specific to VS1 and a lesser but still considerable response to VS2. This pattern was repeated when sheep were vaccinated with tMOMP, which induced a measure of protection. Less efficacious vaccines (mMOMPs) appeared to induce lower seroconversion. No distinction could be made from this data as to whether the abortions which occurred were due to sub-optimal levels of VS1- or VS2- specific antibodies.

Analysis of the protective capacities of VS1- and VS2-specific antibodies, was made possible using passive transfer (Model 2). Antibodies against these regions were first isolated by affinity chromatography. This technique generated two populations of antibodies with different affinities for each peptide. Incorporation of both populations into a single preparation, resulted in significant reduction in levels of splenic infection by Model 2 for both peptide VS1 ($p<0.05$) and VS2 ($p<0.001$). The concentration of anti-VS1 antibodies was probably less than for VS2 in the

inoculation mixtures, so levels of protection were not directly comparable, merely indicative of protective potential. Both VS1 and VS2 were thus shown to confer a significant degree of protection in Model 2. Interestingly, when antibodies of low affinity were used in a similar experiment, no protection was evident; in fact significant increases ($p<0.01$) in both liver and spleen infection were seen in both cases. Whilst naive serum is known to opsonise chlamydia and increase its uptake into host cells, the phenomenon described here implicates surface immunoglobulins in attachment and internalisation of chlamydiae into cells.

A range of MAbs against *C. psittaci* was examined for protective capacities using Model 2. Two MAbs against VS2 were included, one of isotype IgG2b (4/11) and the other IgG3 (4/17). Only MAb 4/11 was protective ($p<0.05$). Furthermore, a blocking ELISA involving MAb 4/11 and affinity purified polyclonal sheep antibodies against VS2 showed clear competition between the two, indicating the production by sheep of antibodies against the same epitope as that recognised by 4/11.

## 8.7. Discussion.

Chapters 4, 6 and 7 demonstrated that passively-transferred antibodies were able to protect mice against hepatic and splenic infection with *C. psittaci* following i.p. challenge. The ovine sera used were obtained from ewes convalescent after experimentally induced OEA and therefore comprised a polyclonal population of *C. psittaci*- specific antibodies. In general, seroconversion does not correlate with protection when measured against the whole *C. psittaci* organism. It was the purpose of experiments in the current chapter to identify antibodies which caused a reduction in chlamydial infection in the passive mouse model and to identify antigenic regions involved in this protection.

The predominant anti-chlamydial immunoglobulin isotype present in both post-vaccination and convalescent sheep sera was shown to be IgG1. This in agreement with the results of Schmeer *et al.* (1992), who found species differences in the predominant antibody isotype secreted following infection with *C. psittaci.* Bovine animals produced mainly IgG2 chlamydia-specific antibodies, whilst sheep and goats preferentially produced IgG1. In the present study involving sheep vaccinated with recombinant proteins, no correlation was found between protection and high levels of seroconversion. Conversely, animals which did not seroconvert well after the second vaccination generally aborted, indicating a crucial role for humoral immunity in protection against abortion. To determine if this lack of protection was due to failure to recognise specific epitopes of MOMP, the sera were analysed for antibodies specific to VS1 and VS2. Of the two animals which aborted after vaccination with tMOMP, one animal did not seroconvert to VS1, and the other one did. However, both animals produced VS1-specific antibodies after aborting, indicating that an inherent lack of VS1-specific B-cells was probably not the reason for the lack of responsiveness to vaccination. Interestingly, high levels of VS1-specific antibodies were detected in the convalescent serum samples from animals which lambed as well as those which aborted. In contrast, most sheep which produced live lambs also produced VS2-specific antibodies after vaccination, but this was not followed by a rise in VS2 antibody titres after challenge. This would imply that the recombinant

vaccine tMOMP, contained an epitope of VS1 which was very similar to native MOMP and that VS1-specific antibodies may be of paramount importance in preventing abortion and possibly in maintaining protection against subsequent infections.

*In vivo* neutralisation by polyclonal VS1- and VS2-specific antibodies derived from ovine convalescent sera and MAbs raised against native antigen demonstrated that both regions were involved in protection against chlamydial infection in mice. Surprisingly, infection could be exacerbated by pre-opsonising chlamydiae with "low affinity" polyclonal antibodies against both VS1 or VS2.

Chlamydia opsonic activity by non-protective antibodies has been reported elsewhere, however in the present study the antibodies have been characterised and were shown to be specific for *Chlamydia* spp. It may be that the increased infectivity seen with chlamydiae pre-treated with naive sheep serum may only reflect the more specific association of VS1- and VS2-specific antibodies with the chlamydial outer membrane. Such a phenomenon may have important consequences in nature. Zhong *et al.,* (1994) reported on the pleiotropic nature of some chlamydial epitopes and this appears to be supported by the findings in the present study. It would be useful to quantify the ratio of "high" to "low" affinity VS1 and VS2-specific antibodies, since it is possible that a balance exists under natural conditions between "high" and "low" affinity antibodies which determines the infectivity of chlamydiae. Indeed, the ratio found in ewes during primary infection may be considerably different to levels in ewes experiencing a second challenge. "Low" affinity antibodies may aid the internalisation process of chlamydiae in host cells, however once inside the phagosome these particular antibodies may become dissociated, thus permitting the organism to multiply unhindered. Indeed, VS2-specific polyclonal antibodies competed with a MAb which mapped to a 12 amino acid residue on VS2 (Vretou *et al.*, 1996). This MAb was an IgG2b isotype, which is known to gain access to intracellular habitats via FcγIII receptors. Interestingly, a second VS2-specific MAb did not show any protection against chlamydial infection; this MAb

(MAb 4/17) was an IgG3 isotype. "Low affinity" antibodies competed less well than the "high affinity" fraction, although inhibition of binding of MAb 4/11 still occurred to a considerable degree with both fractions. Thus, the fractions were probably specific to the same epitope on VS2, although the possibility that the effect was due to steric hindrance between two closely sited epitopes should not be ignored.

The VS1-specific fraction was able to limit binding of MAb 4/11 to approximately 40% of its uncompeted capacity, indicating the close proximities of the VS1 and VS2 epitopes, assuming that the preparation was purely VS1-specific antibodies. Such an arrangement may have important consequences in the design of a recombinant vaccine, as discussed by Ward *et al.* (1995). These workers highlighted the effect of structural constraints on the formation of native conformational epitopes in recombinant proteins. Information such as this may be invaluable in designing recombinant vaccines.

# CHAPTER 9

## CONCLUSIONS

## Conclusions

Several laboratory techniques were examined in order to develop a model which would allow accurate analysis of vaccine efficacies, in particular, recombinant vaccines against OEA. Initially, it was shown that good seroconversion to vaccination in sheep with recombinant chlamydial protein constructs did not necessarily result in protection against OEA (Chapter 8). Conversely, lack of seroconversion generally culminated in abortion. Thus, whilst the latter case implied at least a peripheral role for humoral immunity in protection, the former indicated stimulation of a polyclonal population of antibodies of which only a proportion were protective.

Similarly, analysis of the production of IFN-γ by ovine PBLs was unable to distinguish between protected and unprotected animals. Presumably, the lymphocytes which were stimulated were committed to both protective and non-protective antigens. Whereas lymphocyte proliferation assays suffered from cross-reaction possibly with enteric bacterial components (M. C. M$^{c}$Cafferty, PhD thesis 1994), the stimulation of IFN-γ by native EBs or semi-purified MOMP appeared to be specific for *Chlamydia* spp., but was not dependent on the presence of protective epitopes on the immunising antigen. Furthermore, although it was possible to differentiate between sera from post-abortion ewes and from naive sheep using IFN-γ assays (this thesis, Chapter 7; M. C. M$^{c}$Cafferty, PhD thesis 1994), the assay was not effective in determining vaccine efficacy in sheep (Chapter 7).

Presumably, production of IFN-γ occurred due to stimulation of Th1 cells. Mossman and Coffman (1987) categorised murine T-helper cells into two groups; those secreting IFN-γ and IL-2 (Th1 cells) and those secreting IL-4 (Th2). The activation of T-helper cells has been shown to occur in the early stages of infection in mice (Buzoni-Gatel *et al.*, 1987), as has the induction of IFN-γ (M. C. M$^{c}$Cafferty, 1994). However, the continued effect of IFN-γ on controlling infection is more ambiguous. It may be that whilst IFN-γ is able to aid the control of chlamydial infection during

the early stages of infection, immunomodulation of the immune system during pregnancy may abrogate its secretion and other factors may become more dominant during this immunosuppressed period. Later control of infection may be transferred to the Th2 cells, however neither the presence nor the activity of this group of cells was examined in the current set of experiments. It would be interesting to perform a similar assay to test for the secretion of IL-4 (Th2), as well as IFN-γ and to compare results with protection in pregnant ewes.

Immunisation of mice with recombinant antigens produced seroconversion in only one instance, contrasting notably with the situation in sheep. The exception occurred when mice were immunised with peptides of VS1 and VS3 in a combined adjuvanted preparation. VS3 is not normally surface exposed *in situ* (Baehr *et al.,* 1988; Su *et al.*, 1990) and therefore few antibodies specific to this region are detectable in sera from infected animals. Furthermore, production of antibodies against *Chlamydia* spp. has been shown to be T-cell dependent implying that the presence of chlamydia-specific antibodies may have been mediated by T-helper cell epitopes located in one or both of these regions. Presentation of antigen as peptide rather than as intact protein may result in an alternative mechanism of intracellular processing. This could account for the lack of antibody response to whole recombinant protein in mice, whilst individual peptides of MOMP were able to stimulate an antibody response. A less likely explanation, but one that can not be ignored is that the amount of antigen required to stimulate the murine humoral system may only have been sufficient when concentrated into a peptide preparation. Even so, there would appear to be fundamental species difference between sheep and mice in their abilities to recognise T-helper cell epitopes, to stimulate B-cell proliferation or to recognise T-helper cell epitopes on recombinant proteins. Whether this disparity relates to the use of inbred strains of mice remains to be determined. However, there is evidence that the MOMP-specific antibody response, like that against the heat-shock proteins, hsp60 and hsp70, is linked to the H-2 haplotype in mice (Buzoni-Gatel, 1994; Zhong and Brunham, 1992). Why the murine strain used here should respond to native and not recombinant MOMP may be explained by elucidating specific areas in the

recombinant antigen which may differ from the native form. Indeed, such an experiment may indicate more precisely which regions or epitope within MOMP are actually H-2 linked.

Passive transfer was able to overcome some of the problems associated with eliciting antibody responses in inbred strains of mice. This technique, developed in mice, provided a more accurate prediction of protective capacities of recombinant MOMP vaccines in sheep. The most efficacious of the recombinant constructs was shown to be tMOMP. Evidence suggests that this construct may be folded very similarly to native MOMP, re-inforcing the hypothesis that protection is conferred by conformational epitopes on MOMP.

An attempt to identify protective regions within MOMP, revealed protective capacities of both VS1 and VS2. Antibodies purified from immune sheep sera and specific to peptides representing VS1 and VS2 significantly reduced chlamydial colonisation of mouse organs using the passive immunisation mouse model. Furthermore, antibodies in the VS2 specific fraction, competed for an identical epitope to that recognised by MAb 4/11. This MAb has been identified as being specific for a 12 AA sequence in the VS2 region (I. E. Anderson, personal communication) and was also shown to protect mice against chlamydial infection using the passive immunisation mouse model (this thesis). In addition, examination of the binding properties of VS1 and VS2, suggested that these epitopes were probably in close proximity to each other, further enforcing the concept of protective conformational epitopes.

The mechanism by which antibodies specific to VS1 and VS2 may inhibit infection by *Chlamydia* is unclear. In Chapter 5, the physiological events surrounding passive transfer of pre-opsonised *Chlamydia psittaci* into the peritoneal cavities of mice was examined. Initial results indicated that *chlamydiae* could enter specific cells, such as macrophages and polymorphonuclear leucocytes, even when pre-opsonised with immune sheep serum. No colonisation of organs occurred under these

circumstances. Furthermore, at approximately 48h p.i., development of inclusion bodies ceased, contrasting with *Chlamydia* pre-opsonised with naive sheep serum, where progressively larger inclusions were detected up to maturity at 72h p.i.

Thus, it would appear that in this case, neutralisation of infection occurred at some point post-entry of *chlamydiae* into host cells. This pattern of growth and premature cessation of development, could theoretically be caused by depletion of essential nutrients required for completion of the chlamydial life-cycle and/or, accumulation of toxic waste products within the chlamydiae. The question arises as to whether this is the cause of restriction of development and if so, whether it is due to an inability of MOMP to function as a porin and allow the exchange of materials. Gram-negative bacteria are known to contain porins and electron microscope studies have shown that they usually form trimers with sub-unit sizes of between 30,000-50,000 $M_r$. The MOMP of *C. psittaci* has been shown to be composed of multimers (McCafferty *et al.*, 1995) and MOMP is within the molecular size range associated with porins. Bavoil *et al* (1984) suggested that development of the chlamydial life cycle may be related to opening and closing of porin channels. In this way, cross-linking of the di-sulphide bonds could be integral in the process, such that following internalisation of EBs, reducing conditions would theoretically cause relaxation of the di-sulphide bonds. This would result in opening of the pores and initiation of nutrient uptake. When normal pore activity is prevented, the effects on development of the RB could potentially be fatal.

It can not be ignored, however, that MOMP alone may not be sufficient to protect against OEA. However, caution must be employed in order that detrimental components of the chlamydial organism are excluded from a vaccine, the effects of which have been demonstrated with early *C. trachomatis* vaccines.

Epitopes which are thought to stimulate protective humoral immune responses, appear to include those present in VS1 and VS2. However, it has also been

demonstrated that these epitopes may exhibit pleiotropic characteristics, a phenomenon, which further complicates the search for vaccine components.

A feasible proposition may lie in a vaccine composed of MOMP or sub-regions of MOMP, combined with other chlamydial components. The premise here would be to provide greater structural and physiological support to encourage correct folding of the MOMP construct and to elicit a cumulative immune response, stimulated by MOMP and the additional vaccine components. LPS is known to be intrinsically associated with the outer membrane and the effect of this association on the tertiary structure of MOMP may be considerable. It must be noted, however, that such an arrangement may have evolved in order to protect important epitopes, for instance by exhibiting steric hindrance, in which case the benefits to correct folding may be double edged.

The phenomenon of intermolecular T-cell help supports the inclusion of other components of the chlamydial organism in addition to MOMP, in a vaccine. The possibility exists that co-stimulation of the immune system by individual antigens may be a pre-requisite for the development of a protective immune response.

# APPENDICES

(N.B. Concentrations in parentheses refer to stock solutions)

## Appendix I: RPMI Maintenance Medium

| | |
|---|---|
| RPMI (+Glutamine/-Sodium bicarbonate; Flo Laboratories) | 500ml |
| Gentamicin (10mg/ml saline; filter sterilised through 0.2 μm) | 2.5ml |
| Nystatin (10,000 units/ml) | 1.25ml |
| 1M Hepes | 5ml |
| 8% Sodium bicarbonate | 12ml |
| Newborn Calf serum (Heat inactivated) | 25ml |
| Streptomycin (100mg/ml; adjust to pH 6.8 with sodium bicarbonate) | 1ml |

## Appendix II: RPMI Infection Medium

| | |
|---|---|
| RPMI (+Glutamine/-Sodium bicarbonate; Flo Laboratories) | 500ml |
| Gentamicin (10mg/ml saline; filter sterilised through 0.2 μm) | 2.5ml |
| Nystatin (10,000 units/ml) | 1.25ml |
| 1M Hepes | 5ml |
| 8% Sodium bicarbonate | 12ml |
| Newborn Calf serum (Heat inactivated) | 10ml |
| Streptomycin (100mg/ml; adjust to pH 6.8 with sodium bicarbonate) | 1ml |
| Cycloheximide | 1μg/ml |

**Appendix III: Chlamydia Transport Medium (CTM)**

| | |
|---|---|
| Sucrose | 149.2g |
| Potassium di-hydrogenase Orthophosphate ($KH_2PO_4$) | 1.024g |
| D-Potassium hydrogen Orthophosphate ($K_2HPO_4$) | 2.474g |
| L-Glutamic Acid | 1.442g |
| 0.4% Phenol Red | 8ml |
| Dissolve in reagent grade water | 2000ml |

Add:-

| | |
|---|---|
| Foetal Bovine serum | 200ml |
| Nystatin (10,000 units per ml) | 30ml |
| Gentamicin (10mg/ml saline; filter sterilised through 0.2 μm) | 10ml |
| Streptomycin | 0.2g |

Final pH 7.2 (Adjustment should not be necessary). Filter sterilise through 0.2 μm. Store at –20° C.

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Dr M. Norval

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